厚生労働行政推進調査事業費補助金

化学物質リスク研究事業

OECDプログラムにおいてTGとDAを開発するためのAOPに関する研究
(H30-化学-指定-003)

令和元年度　総括・分担研究報告書

研究代表者　小島　肇

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OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

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研究要旨

動物実験 3Rs の国際的な浸透に加えて、実験動物とヒトとの種差等の克服のために、既存の毒性試験法の見直しが進んでいる。経済協力開発機構（OECD: Organisation for Economic Co-operation and Development）においても、反復投与毒性、生殖発生毒性、感作性、発がん性などの有害性発現経路（AOP: Adverse Outcome Pathway）を開発し、動物実験代替法（以下、代替法）を念頭においた試験法ガイドライン（TG: Test Guideline）の公定化や *in silico* 法の確立に AOP 情報を活用している。一方で、毒性情報を網羅した IATA（Integrated Approaches to Testing and Assessment）を開発し、それに基づく DA（Defined Approach）により化学物質の安全性評価を推進する戦略がある。DA とは、単独の代替法ではなく、種々の試験データを組み合わせて化学物質の全身毒性を把握しようとする試みであり、OECD では DA の行政的利用が検討されている。このような国際的な潮流に乗り、日本が得意とする分野で主導権を握って、AOP や TG を公定化し、さらには IATA や DA の TG の開発及び普及に協力することが本研究班の目的である。

昨年度からの継続した活動の中、本年度に以下に示す 3 試験法が OECD の TG として 2019 年 6 月に採択された。

1）光安全性　活性酸素種（ROS：Reactive Oxygen Species）アッセイ（TG495）
2）皮膚感作性試験代替法　アミノ酸誘導体反応試験（ADRA：Amino acid Derivative Reactivity Assay）（TG442）
3）培養表皮モデル LabCyte EPI-MODEL24 を用いる腐食性試験代替法（TG431）

AOP に関しては、OECD において外部 peer reviewer の選考が難航し、“Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response”の審議が遅れ、現在も進行中である。本年度中の日本発の AOP の採択は叶わなかったが、来年度採択に目途が立った。

厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）
OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

令和元年度 分担研究報告書

AOP、TG、DAの開発、AOP国内マニュアルの作成

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研究要旨

動物実験 3Rs の浸透に加えて、実験動物とヒトとの種差等の克服のために、既存の毒性試験法の見直しが世界的に進んでいる。経済協力開発機構（OECD: Organisation for Economic Co-operation and Development）においても、全身毒性試験（免疫毒性、生殖発生毒性、発がん性及び光安全性等）の有害性発現経路（AOP: Adverse Outcome Pathway）を開発し、その情報を活用して動物実験代替法を念頭においた試験法ガイドライン（TG: Test Guideline）の公定化や *in silico* 法を確立する一方で、AOP 等の毒性情報を網羅した IATA（Integrated Approaches to Testing and Assessment）を開発し、それに基づき、TG と同格の扱いになる DA（Defined Approach）による化学物質の安全性評価を推進している。このような国際的な潮流に乗り、日本が得意とする分野で主導権を握って、AOP や TG を公定化し、さらには IATA や DA の開発に協力することが本研究班の目的である。

昨年度からの継続した活動の中、本年度に以下に示す 3 試験法が OECD の TG として採択された。本年度、AOP の採択は叶わなかったが、AOP154 案“Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response ”に関しては、来年度採択に目途が立った。

1）光安全性　活性酸素種（ROS）アッセイ（TG495）
2）皮膚感作性試験代替法 アミノ酸誘導体反応試験（ADRA）（TG442）
3）LabCyte EPI-MODEL24 を用いる腐食性試験代替法（TG431）

研究分担者氏名・所属研究機関名及び
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尾上誠良　静岡県立大学　薬学部・薬剤学分野　教授
笠原利彦　富士フイルム株式会社　安全性評価センター技術マネージャー
加藤雅一　株式会社ジャパン・ティッシュ・エンジニアリング(J-TEC)主任研究員
木村　裕　東北大学医学系研究科・医学部・皮膚科学分野准教授
久田　茂　日本免疫毒性学会試験法委員会(あすか製薬株式会社)

A. 研究目的

本研究班では、OECD の AOP 開発プロジェクトの中で、化学物質の毒性情報等を集積しながら、免疫毒性、生殖発生毒性、発がん性及び光安全性等に関する日本発の AOP 開発を進める。既存の AOP 情報をもとに開発された皮膚感作性試験代替法 ADRA（Amino acid Derivative Reactivity Assay)、免疫毒性試験 MITA(Multi-Immuno Toxicity Assay)、発生毒性試験スクリーニング Hand1-Luc EST(Embryonic Stem cell Test)、光安全性試験スクリーニング ROS（Reactive Oxygen Species）アッセイ、LabCyte EPI-MODEL24 を用いる腐食性試験代替法については、試験法毎に独立した国内外の専門家による第三者評価(peer review)を受けた後、TG を開発する。一方で、皮膚感作性 DA(DASS: Defined Approach for Skin Sensitisation)の開発に関与することを通じて、IATA や DA の国内での普及に務める。

B. 研究方法

B.1. AOP、TG、DA の開発、AOP 国内マニュアルの作成

B.1.1. AOP 国内マニュアルの作成

OECD の AOP 開発プロジェクト EAGMST（Extended Advisory Group on Molecular Screening and Toxicogenomics)及び、TG の開発プロジェクト WNT（Working Group of the National Coordinators of the Test Guidelines Programme)の進捗に合わせ、班員を支援した。

OECDの定める「AOPの開発・評価に関するガイダンス　No.184(以下、AOPガイダンスと記す)」を翻訳し、マニュアル作成に向けた資料整備を進めた。

B.1.2. AOP開発

AOP に関しては、日本免疫毒性学会会員をメンバーとする同学会試験法委員会 AOP 検討小委員会に免疫毒性 AOP の開発を委託している。

文献調査の結果に基づいて、カルシニューリン阻害を分子初動(MIE: Molecular initiating event)とし、T 細胞依存性抗体産生抑制(TDAR)を有害性発現(AO：Adverse Outcome)とする AOP154 案“Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response”を作成し、peer review に対応した。

B.1.3. TG 開発

日本から提案している試験法である皮膚感作性試験代替法 ADRA、光安全性試験 ROS

アッセイ(尾上分担研究者との協同研究)、LabCyte EPI-MODEL24 を用いる腐食性試験代替法の TG 採択のために、WNT や専門家会議にて交渉した。また、ヒトアンドロゲン(AR)受容体安定トランスフェクト転写活性試験(AR STTA: The Stably Transfected Transactivation method using the AR-EcoScreen™ cell line)TG458 の改訂に向け、尽力した。

分担研究者の相場が開発し、他の研究班でバリデーションを終了させた IL-2 を指標とした免疫毒性試験の TG を目指し、海外の専門家を招聘したバリデーション報告書の peer review を進めた。

また、*in vitro* 免疫毒性試験に関する Detailed Review Paper(DRP)の作成を国際専門家とともに進めた。

B.1.4. DA の開発協力

足利分担研究者とともに、OECD 専門家会議において、DASS の開発に協力した。

C. 研究結果

C.1. AOP、TG、DA の開発、AOP 国内マニュアルの作成

C.1.1. AOP国内マニュアルの作成

AOP ガイダンスを翻訳して、班員に普及した(添付資料 1)。今後、HP などで公開するとともに、来年度のマニュアル作成に反映させる予定である。

C.1.2. AOP開発

AOP154 案“Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response”に関しては、2018 年 6 月に EAGMST の内部 peer reviewer が終了したものの、外部 peer review の開始まで 1 年もまたされた。この理由として、OECD 事務局が外部専門家との連携関係を模索している最中であることが挙げられる。何とか、免疫毒性の専門家グループも見つかり、2019 年末より、外部 peer reviewer による評価が進んでいる。2020 年 6 月の EAGMST 会議で採択されるよう免疫毒性学会の皆様に尽力して頂いている。

C.1.2. TG の開発

1) 皮膚感作性試験

皮膚感作性試験代替法 ADRA は、2019 年 6 月 18 日の OECD TG442C(添付資料 2)として採択された。

ただし、その後、記載ミスが見つかったこと、新たな項目を追加したいとの富士フイルムの意向を受けて、11 月に TG の改定を求める SPSF を提出し、2020 年 4 月の WNT で作業計画に採択された。

2) 光安全性試験

ROS アッセイの TG は、2019 年 6 月 18 日の OECD TG495(添付資料 3)として採択された。

引き続き、ROS アッセイを含む IATA 開発に関する SPSF を 11 月に提出し、2020 年 4 月の WNT で作業計画に採択された。

3)腐食性試験

LabCyte EPI-MODEL24 を用いる腐食性試験代替法は、2019 年 6 月 18 日の OECD TG431 培養表皮モデルを用いた腐食性試験(添付資料 4)の中の一つに採択された。

4) 内分泌かく乱試験

AR-STTA TG458の改訂に向け、追加される2試験法 The AR-CALUX® method using the AR-CALUX® cell line 及び The ARTA method using the 22Rv1/MMTV_GR-KO cell lineのpeer reviewerを務めた。

結果として、2020 年 4 月の WNT で本改定 TG458 が採択された。

5) 免疫毒性試験

表 1 に示す海外の専門家を招聘し、IL-2 Luc アッセイバリデーション報告書の peer review を進め、評価書案(添付資料 5)が最終段階にある。2019 年 2 月の対面会議後、バリデーション報告書の改訂を受けて、2019 年 10 月および 11 月の電話会議を経て(添付資料 6 および 7)作成された。ただし、まだ最終合意には至っていない。

本件に関しては、2019年11月にIL-2 Lucアッセイの TG開発のSPSFを提出した。ただ、2020年4月のWNTにおいてプロジェクトとしての採択は認められなかった。まずは以下に示すDRPの提出を急ぐべきとの忠告を受けた。来年の再提出を目指し、DRPを開発するとともに、評価報告書とバリデーション報告書を確実に作成することを目指していく。

一方、相場らの開発した*in vitro*免疫毒性試験を円滑にTGに導くための準備として、OECDに了承されたDRPの開発を表2に示す国際的な専門家の協力を得て、進めている。2019年10月の電話会議を経て（添付資料8）、2020年1月には専門家を招聘して、対面会議を行い（添付資料9）、表3に示す目次（Table of Content）に合意を得るとともに、項目毎の担当部分について意見交換した。

来年度早々、DRPをOECDに提出する予定である。

C.1.3. DA の開発協力

OECD 専門家会議（電話会議）で DASS の開発に寄与した。2019 年度だけでも、10 回の電話会議が開催されているが、議事録の一部を添付とした（添付資料 10）。

ヒト及び動物実験結果の再評価、適用範囲および不確定因子について、それぞれサブワーキンググループの討議が一年に渡って続いており、その提案を待って DASS の最終的な議論が 2020 年度になされる予定である。

D. 考察

D.1. AOP の開発

本研究班から提案している免疫抑制の AOP154 案 ”Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response”は OECD における外部 peer review が進行中である。ただ、現在の状況から来年度採択に目途が立ったと考えている。

D.2. TG の開発

皮膚感作性試験代替法 ADRA、光安全性 ROS アッセイ及び LabCyte EPI-MODEL24 を用いる腐食性試験代替法に関しては、2019 年 6 月 18 日に TG として採択された。

一方、新たに 3 件の SPSF を OECD に 2019 年 11 月に提出し、2020 年 4 月の WNT で OECD 作業計画に加えられることになった。継続して、TG の開発および修正に関与していきたい。

D.3. IATA 及び DA の公定化

DASS に関する OECD 活動に対し、引き続き協力していく予定である。来年度は光安全性 IATA の開発を国内外の専門家とともに進め、動物実験を用いない光安全性評価の体系化を日本からも提案していく予定である。

E. 結論

昨年度からの継続した活動の中、本年度 3 試験法が OECD の TG として採択された。本年度、AOP の採択は叶わなかったが、AOP154 案“Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response ”に関しては、来年度採択に目途が立った。

引き続き、OECD の活動の中で、日本が得意とする分野で主導権を握って、AOP や TG を公定化し、さらには IATA や DA の開発に協力していく予定である。

F. 添付資料

1. AOPの開発・評価に関するガイダンス No.184

2. OECD Test Guideline 442C for the Testing Chemicals on in chemico skin sensitisation assays addressing the Adverse Outcome Pathway Key Event on covalent binding to proteins
3. OECD Test Guideline 495 for the Testing Chemicals on ROS (Reactive Oxygen Species) assay for phototoxicity
4. OECD Test Guideline 431 for the Testing Chemicals on *in vitro* skin corrosion, Reconstructed human epidermis test methods
5. IL-2 Luciferase (Il-2 Luc) Assay Report of the Peer Review Panel.
6. Teleconference for IL-2 PRP October 1, 2019
7. Teleconference for IL-2 PRP, November 11, 2019
8. Teleconference for DRP on in vitro immunotoxicity, October 28, 2019
9. 2020年1月DRP開発のための対面会議議事録
10. Expert Group on DASS, November 18& 19

表 1. IL-2 Luc アッセイ第三者評価委員会メンバーリスト

No.	Name	Affiliation	Country
1	Henk van Loveren	Maastricht University	Netherlands
2	Haley Neff-LaFord	Seattle Genetics, Inc.	USA
3	Barbara Kaplan	Mississippi State University	USA
4	Fujio Kayama	Jichi Medical University	Japan
5	Xingchao Geng	National Center for Safety Evaluation of Drugs (NCSED)	China
6	Sang-Hyun Kim	Kyungpook National University	Korea
7	Takao Ashikaga	National Institute of Health Sciences	Japan

表 2. In vitro 免疫毒性試験　総説（Detailed Review Paper）作成メンバーリスト

No.	Name	Affiliation	Country
1	Emanuela Corsini	Università degli Studi di Milano	Italy
2	Dori Germolec	NTP/NIEHS	USA
3	Barbara Kaplan	Mississippi State University	USA
4	Henk van Loveren	Maastricht University	Netherlands
5	Haley Neff-LaFord	Seattle Genetics, Inc.	USA
6	Erwin L. Roggen	3RsMC ApS	Denmark
7	Setsuya Aiba	Tohoku University	Japan
8	Yutaka Kimura	Tohoku University	Japan
9	Takayuki Yoshimoto	Tokyo Medical University	Japan
10	Hajime Kojima	JaCVAM, National Institute of Health Sciences	Japan

表 3.　Table of Content (ToC) for DRP

Item
ABOUT THE OECD FOREWORD
LIST OF ABBREVIATIONS
EXECUTIVE SUMMARY
I. Introduction
II. Background
III. Basic concept of immunotoxicity
IV. State-of-the-art of AOP on immunotoxicity
V. State-of-the-art in the field of *in vitro or non-animal* assay
VI. Performance factors of *in vitro* assay
VII. Assay qualification information of *in vitro* assay
VIII. Selection factors for the reference compound developing in vitro assay
IX. Reference compound list
X.　Battery of *in vitro or non-animal* assays
XI. Discussion and Recommendation
XII. References
XIII. Appendix

G. 研究発表

G-1.学会誌・雑誌等における論文一覧

（国内誌　1　件、国際誌　7　件）

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H. 知的所有権の取得状況

H-1. 特許取得

特になし

H-2. 実用新案登録

特になし

H-3. その他

特になし

経済開発協力機構

English, French

環境局
化学品委員会と化学品・農薬・バイオテクノロジーに関する調査委員会の合同会合

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AOPの開発・評価に関するガイダンス文書（改訂）
リスク管理シリーズ
No. 184

ガイダンス文書第2版、2013年初版の差し替え

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リスク管理シリーズ

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有害性発現経路（AOP）の開発・評価に関するガイダンス文書（改訂）

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

環境局
経済協力開発機構
2017年　パリ

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本書は化学物質の適正管理のための国際機関間プログラム（IOMC）の枠組みで作成された。内容は、必ずしも個々のIOMC参加機関の見解または規定されたポリシーを反映するものではない。

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有害性発現経路（AOP）の開発・評価に関するガイダンス文書

（第二版）

緒言

有害性発現経路（AOP）方法論は、化学物質の生物学的および毒物学的影響に関する関連情報を収集、整理、評価するための枠組みを提供する手法である。より具体的には、AOP手法は、生物学的組織の分子レベルの摂動と規制上の懸念のある生物学的組織のレベルでの有害性発現との間で、生物学的に妥当で経験に支えられる関連する既存の知識を体系づける。この手法は、化学物質や他のストレス因子の悪影響を理解するため、作用機序（MOAおよび/または作用機構）により支えられている。このガイダンス文書は、関連する科学データと結果として得られる知識の特定と使用を含む、語彙、概念、およびAOPの開発に関する洞察を紹介する。また、本書では、AOPの潜在的な規制上の使用についても簡単に説明している。文書の最後には、AOPの概念と構成物、およびその最終的な適用理解の促進を目的とした用語集がある。

補足文書であるOECD AOPユーザーハンドブックは、AOP開発に関する詳細な情報を提供し、AOPの構築方法の詳細を探している人にとってより適切な文書である（OECD, 2016a）。 AOPユーザーハンドブックには、基礎となる情報の信頼性を評価するためのガイダンスも含まれている。試験法と評価への統合アプローチ（IATA）の開発におけるAOPの使用に関するOECDガイダンス文書（OECD, 2016b）は、AOPの概念をさまざまな目的でIATAを開発するための枠組みとして適用する方法を説明している。 文書化されたAOPの数が増えるにつれて、IATAでの適用とさまざまな規制状況での使用について、さらにデモを行い、ガイダンスを作成できる。 経験が増えるにつれて、AOPと調和したIATAに基づくIATA開発のためのガイダンス文書が開発されることが期待される。

このガイダンスは、2012年12月にOECDのExtended Advisory Group on Molecular Screening and Toxicogenomics（EAGMST）と共同で事務局によって作成された。当時、専門家グループと加盟国がAOPの開発と評価の経験を積み、ガイダンスを改訂する必要性が認められた。それ以来、AOPの開発である程度の経験が得られ、AOPの開発と評価のためのガイダンス文書のユーザーハンドブック補足が公開された。ユーザーハンドブックには、AOP雛形（元のガイダンスのパートII）と、データの要約、AOP評価、および元のガイダンスからのAOPの信頼性を扱う項目が記載されている。ユーザーハンドブックは、AOP-Wikiと証拠の重み付け（WoE）の考慮事項に関連する資料を含むように開発されたものであり、必要に応じて定期的な更新が必要になる。これとは対照的に、現在のガイダンスは、AOP枠組みにおいて変更される可能性が低い領域を扱っている。このガイダンス文書の第2版は、AOP開発プログラムの歴史的背景を提供し、AOPの構築に必要な要素とAOP枠組みの原則の概要を示している。

ガイダンス文書の第2版は、2017年4月の第29回WNT会議で承認された。本書は、化学物質委員会とOECDの化学物質、農薬、バイオテクノロジーに関する作業部会の合同会議の責任で発行される。

目次

背景

1. 化学物質の悪影響からヒトと環境を保護するための歴史的な枠組みは、主に懸念のある単一の化学物質による動物個体を用いた毒性試験に集中している。ただし、コストと時間がかかるため、ヒトと生態系に悪影響を与える可能性のあるすべての化学物質を網羅的に試験することは実用的でも実行可能でもない。現実的には、化学物質の悪影響をより効率的に予測するための科学的に防御可能なモデルとツールが必要性とされている。現在まで、生物学的システムに関する私たちの限られた知識は、効果の外挿の基本とした作用機構情報を使用する努力を妨げてきた。それにもかかわらず、トキシコゲノミクス、バイオインフォマティクス、システム生物学、計算毒物学の進歩は、本書にてその可能性を提供している（NRC, 2007; Krewski et al, 2010）。有害性発現経路（AOP）は、関連する化学的、生物学的および毒物学的情報を収集して評価し、より効率的で予測的な評価および試験戦略の推進を支援する枠組みとして提案されている。この枠組みは、有害作用への経路をよりよく理解するために、作用機序（MOAおよび/または作用機構）の利用により支えられる基礎情報で編成されている。既存の方法と新しい方法をシステム生物学にリンクする。簡単に言えば、AOPの証拠の重み付け（WoE）の検討は、Meek et al, 2014aで報告されたように、国際的に多数の科学者が関与する作用機序（MOA）/人間関連分析の既存の進化する枠組みに組み込まれた概念と原則に基づいている。

2. このガイダンスの主な目的は、AOPの開発・評価、およびAOP固有の用語の定義を含む一貫した情報収集と組織化の枠組みを紹介することである。 これに関連して、AOPは、規制上の決定に関連する組織の生物学的レベルでの分子初動事象（MIE）と最終的な有害性発現（AO）との間の因果関係の経路に関する既存の知識を記載した概念的な構成体である（Ankley et al, 2010）。

はじめに

3. 毒物学的評価のための現在の試験手法の限界と、新しい生化学および細胞試験システムと計算予測方法の急速な発展を認識し、規制当局およびその他の利害関係者は、効率と予測可能性を高めるための試験法と評価の戦略を進化させるための基礎として、代替法を含む*in vivo*試験からの既存の知識とその他の情報源を統合する方法を模索してきた。

4. 過去20年にわたり、さまざまなグループが、毒性物質が公衆衛生に関与するAOを誘発するプロセスを定義するためのシステムおよび経路による手法を提唱してきた。経路手法の初期では、曝露用量反応モデルまたは生物学に基づいた用量反応モデルと呼ばれることが多かった（Clewell et al, 1995; Shuey et al, 1995）。 2001年、MOA情報を使用して動物データとヒトとの関連性を判断するための枠組みが、代謝から影響への一連の主要事象による経路の分類に基づき、国際化学安全プログラム（IPCS）（Sonich-Mullin et al, 2001）によって発表された。後者は2002年にOECDによって採択された（OECD, 2002）。 2007年、米国科学アカデミー（NAS）は、同様の原理に基づく「毒性経路」の概念が提案された21世紀の毒性試験の展望と戦略に関する報告書を発表した（NRC、2007）。報告書には、好ましくはヒト由来の細胞を使用し、適切に設計された*in vitro*法での化学物質曝露による生物学的経路の摂動を評価する毒性試験の方向を変える展望が含まれていた。

5. 2006年、2007年、2008年に予測毒性に関するMcKimカンファレンス（http://mckim.qsari.org）以来、IPCS MOA枠組みの改良（Boobis et al, 2006; 2008）と並行して、「有害性発現経路（AOP）」が進化した（http://mckim.qsari.org）。もともとはAnkleyとその同僚によって導入されたものであり（Ankley et al, 2010）、OECDプログラムの状況下で、規制の決定に関連する組織の生物学的レベルでのMIEと最終AOの間の因果関係の経路に関する既存の知識を構成する概念として発展した。AOPでは、さまざまな出展から既知の情報を統合することが重要である。この手法は、化学物質が最初に到達し、生物体の最初の標的と相互作用することで毒性が生じるという概念に基づいている。そのため、AOPは、MIEから対象のin vivo転帰への事象の連続的に進行する（図1）。一般的に、次のような広範な経路のセットを指す：1）ストレッサーと生物学的目標（例えば、DNA結合、タンパク質酸化など）との相互作用から生じる摂動を表すMIEから始まる。2）毒性の進行に不可欠な一連の生物学的活動（例えば、遺伝子活性化、または組織発達の変化など）に続く; 3）最終的に、人間または生態系のリスク評価者に関連する最終的なAO（例：死亡率、生殖障害、がん、または絶滅など）に到達する(OECD 2011、ENV / JM / MONO(2011)8)。

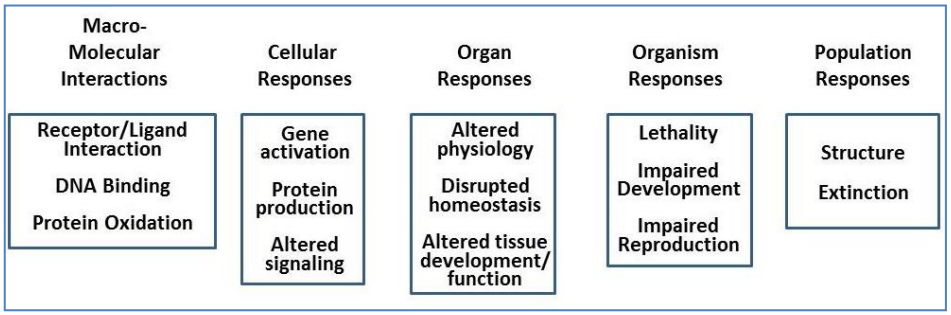

Figure 1. 有害性発現経路（AOP）の概略図は、生物学的組織のさまざまなレベルでのいくつかの例を参照して説明されている。

6. AOPの概念は、2009年に環境毒性および化学協学会によって開催されたPellston会議など、近年の多くの議論に基づいて進化している（Villeneuve and Garcia-Reyero、2011; Watanabe et al.,2011; Perkins et al, 2011; Nichols et al., 2011; Celander et al., 2011; Kramer et al., 2011; ENV / JM / MONO（2012）10 / PART1; ENV / JM / MONO（2012）10 / PART2 ; Enoch and Cronin 2010; ENV / JM / MONO（2011）6; Schultz et al., 2011; Hill,1965; US EPA, 2005; US EPA, 2011）。 これらの議論から作成された論文は、既存のデータからの導出や、ゲノミクスデータからのAOPの逆行工学の手法など、AOPのさまざまな側面を扱っている。 OECDが主催する「化学物質分類における機械的情報の使用」という題名のワークショップが2010年12月にワシントンDCで開催され、短期的に（その後2年間）多くの勧告と結論が出された。 これらの推奨事項は次のとおり：

1) 確立された有害性作用（皮膚感作性など）のAOPを開発する際には、相互作用を促進するために、毒物学者や他の科学者をAOPの議論に参加させる
2) 2010年12月のワークショップで始まった概念実証を、いくつかの異なる長期的なヒトの健康および生態毒性指標のAOPを開発することにより完成させる
3) 以下の開発を含む、AOPを特定、評価、および推進し、OECD QSAR Toolboxに統合するための戦略計画を開発する
    a) AOPの開発と評価に使用できる情報雛形
    b) AOPの完全性と受け入れを評価するための一連の指針
    c) AOPの相互承認を達成するための書式
4) AOPに関連する用語を調和させる (OECD 2011, ENV/JM/MONO(2011)8).

7.（1）と（2）の提言に対応して、OECDは、皮膚感作を引き起こすタンパク質結合のAOPを開発した。 図2は、タンパク質への共有結合によって開始される皮膚感作に関連する経路の流れ図を示している（OECD 2012, ENV / JM / MONO(2012)10 / PART1; ENV / JM / MONO(2012）10 / PART2）。 このAOPの開発で得られた経験と提言への取り組みに基づいて（3）と（4）、現在のガイダンスの初期版が開発された。 この初期のガイダンスは、AOPの開発と評価に不可欠な要素と原則を示すだけだったが、本書にて改良された。 詳細はユーザーズハンドブックに記載されている。このハンドブックは、AOP開発者と評価者から受け取ったフィードバックに基づいて、このガイダンスよりも簡単に更新できるように作成されている。

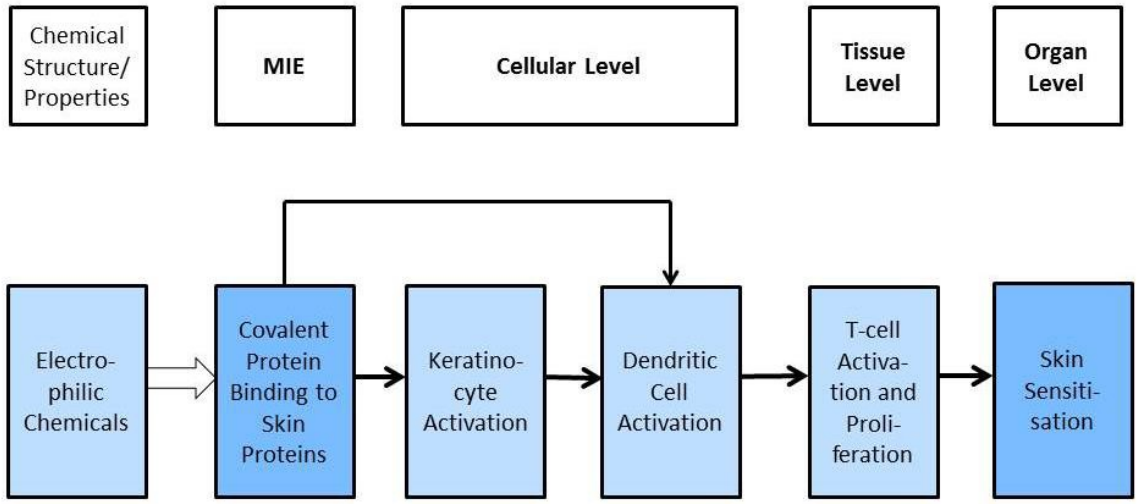

Figure 2. 皮膚感作に関するAOPの例 (出典 OECD 2012, ENV/JM/MONO(2012)10/PART1).

8. AOPの分野での国際的な共同作業を支援するOECD Extended Advisory Group on Molecular Screening and Toxicogenomics（EAGMST）のメンバーは、2014年の国際ワークショップ（http：//www.saaop.org / workshops / somma.html）に参加した。 その結果である論文は、AOPの開発と評価の概念的側面の改良に貢献した（Villeneuve et al,2014a,b; Perkins et al,2015; Garcia-Reyero,2015; Becker et al.,2015; Groh et al,2015a,b; Tollefsen et al.,2014）。 論文の一つ（Villeneuve et al.,2014a）には、AOP開発を導く5つの基本原則が説明されている。

(1) AOPは化学的特異性ではない

(2) AOPは組み換え可能な構成方式であり、主要事象（KE）および主要事象の関係（KER）という名前の再利用可能な要素で構成されている

(3) KEとKERの単一軸で構成される個々のAOPは、AOPの開発と評価の実用的な単位である

(4) 共通のKEとKERを共有する複数のAOPで構成される連絡網は、ほとんどの実際のシナリオの予測機能単位となる可能性がある

(5) AOPは、新しい知識が生成されるにつれて時間とともに進化する生きた文書である

9. 別の論文では、ユーザーズハンドブックに含まれる修正されたBradford-Hillの考慮事項（Becker et al, 2015）に基づいて、AOPの成熟度と信頼レベルの評価の基礎としてWoE分析の事例を示した。 これらの経験から得られた主要な教訓も文書化され、事例と合わせて、AOPの適用に対する信頼性を高めるために必要な文書の性質と形式の共通理解を深めることに寄与している。

10．AOPは単一の軸（たとえば、生物組織のレベル; 図1参照）で表されるが、毒性は多次元（たとえば、性別、種、年齢）であるため、MIEと最終AOの間の経路はかなり異なる。これは、複数の臓器相互作用（例：皮膚感作）、複数の事象（例：反復投与毒性）、経時的蓄積（例：神経毒性）の結果である、より「複雑な」長期指標に特に当てはまるか、または生物の特定の生命段階に関連している（例：発生毒性）。それにもかかわらず、毒性反応を実現するには多くの生化学的段階が必要であるが、MIEはその後のすべての段階の前提条件である（Enoch and Cronin, 2010）。そうは言っても、単一のMIEがいくつかの多段階のシグナル伝達に影響を与える可能性があると理解されており（例えば、Casperse-3のタンパク質発現の減少、Casperse-6の同時活性化）、そのうちの1つ以上が特定のAOに寄与する可能性がある。さらに、AOPは、生物レベル全体ではなく分子レベルで化学的相互作用が始まるという事実に基づいている。したがって、*in vivo*で観察されるAOは、化学物質と内因性生体分子との相互作用によって引き起こされる多段階の生物学的な結果である。

11. AOPは生物学の実用的な単純化であるが、AOPネットワークで表すことができるより広範な状況を発生させると認識できる。 特定のMIEはいくつかの最終結果につながる可能性があり、逆に、いくつかのMIEは同じ最終結果につながる可能性がある。 ただし、AOPは、一つのMIEと一つの最終AOのみに焦点を当てた評価するように設計する必要がある。

12. AOPの各構成物自体は、モデル化されている生物学的システム内で進行している他の経路の影響を受ける場合がある。 さらに、AOPユニットを構成するKEの非分岐の連続は、他のAOPと共有されるKEまたはKERである可能性が高く、AOPネットワークの作成につながる相互作用をつくりうる。 AOPネットワークとして知られるこれらの相互に作用するAOPのシステムは、ほとんどの実際のシナリオをより代弁していると考えられている（Villeneuve et al, 2014a; Knapen et al, 2015）。 AOPは、AOにつながるKEの単一の線形連続として視覚化できるが、AOPネットワークはより広範な画像を提供し、同じAOの発現につながる可能性のある摂動の可能性があるさまざまな利用可能なKEおよびKERを含む。 これらのより複雑なネットワークは、AOP知識根拠（KB）（後述）にある他のツールを使用して表示される。 これらのネットワークは、たとえば、ある器官（脳）のMIEが別の器官（生殖腺）のAOとしてどのように現れるかを示す。

13. OECDは、AOPの開発と適用を可能にするITツールの構築に取り組みんでいる。 AOP-KBは2014年9月25日に開始され、いくつかの基本単位で構成されている。 AOP-Wikiは、KEの共有とAOP開発者間の協力を促進する、開発済みまたは構築中のAOPの中央貯蔵スペースとして機能するオープンソースインターフェイスである。 AOP XPlorerは、AOPネットワークの視覚化を支援するAOP-KBの追加基本単位である。 Effectopediaは、KE間の定量的反応-反応関係、KEの測定に利用できる試験法および生体指標などの追加の構造化情報を把握できる。意思決定段階を促進するために、グラフィカルインターフェイスを介してこの情報を表示する。 4番目の基本単位である中間効果データベース（IEDB）は、AOP-KBを、典型的な化学物質、つまり特定のAOPにつながる科学的推論を裏付ける化合物を使用した*in vitro*アッセイの実際の試験結果につながる。 IEDBはIUCLID事例であり、ほとんどがOECD Harmonized Template（OHT）201を使用する。これは、非古典的な試験方法を対象としている。第三極のIUCLIDシステムからのOHT 201データは、IEDBに簡単に転送できる。 e.AOP.Portalは、AOP-KBの主要な導入点であり、AOP-WikiおよびEffectopediaで現在利用可能なAOPを見つけるための検索メカニズムを提供している。 e.AOP.Portalは、OECD作業計画にあるすべてのAOPの状態を提供する。公開されたすべてのAOPは、この同じサイトからアクセスできる。

14. AOPおよびAOP KBの開発と広報により、さまざまな規制活用が可能になると予想される。たとえば、上記の2014年のワークショップの一環として、Groh et al.（2015a）はAOの予測能を向上させることを目的とした研究を進めるために、AOP概念をどのように使用できるかを調査

した。 さらに、魚の成長を事例研究として使用し、AOP概念を使用して、既存の知識と潜在的な代替試験のギャップを確認するため、特定の慢性毒性事例に利用可能な知識について高い評価を得た（Groh et al, 2015b）。 また、ワークショップグループは、さまざまな規制目的でIATAの開発を通知する際のAOPの役割の解明に焦点を当てており（Tollefsen et al, 2014）、AOPの開発の相対的な程度と科学的信頼度に応じて、AOPのさらなる規制適用の可能性を調査した（Perkins et al, 2015）

15. 最初から、目的の十分性を判断するために定義とチェックリストおよび/または評価枠組みが必要である。開発のあらゆる段階のすべてのAOPが役立つ。ただし、特定の規制状況でAOPを信頼できる範囲は、その開発レベル、許容できる不確実性の段階、および証拠の段階（たとえば、情報とデータの詳細、品質、および量）に関連しており、利用可能である（ユーザーズハンドブックを参照）。 OECDは、AOP-Wikiに入力されたAOPのガイダンス原則、完全性、および科学的堅牢性の順守を評価するために、特定のAOPに関して提出された提案を作業計画に承認する主な責任を持つEAGMSTと連携して機能し、加盟国の承認を求めている。 EAGMSTの承認後、Working Group of National Co-ordinators of the TGs programme（WNT）のワーキンググループとWorking Party on Hazard Assessment（WPHA）は、AOPの承認とその後のJoint Meeting（JM）からの機密解除について責任を負う。

16. この文書の目的は、AOP固有の用語の定義の用語集を含む、一貫した情報収集とAOPへの編成のための枠組みを提供することである。 この文書は、AOPと使用する必要のある用語を開発するために必要な情報の断片についての見通しを示すことを目的としている。 最後に、この文書では、規制状況におけるAOPの潜在的な使用についても簡単に概説している。 IATAにAOPを使用する方法に関するガイダンスが利用可能（OECD, 2016b）だが、リスク評価へのAOPの使用に関するさらなるガイダンスは将来開発される予定である。 AOP WikiでのAOPの開発と実装に関する詳細なガイダンスは、ユーザーズハンドブックに記載されている。これは、得られた経験に基づいて時間とともに進化する現在の文書の補足である。 AOPを開発または評価する場合、現在のガイダンスとユーザーズハンドブックを意味する両方の文書の内容を考慮する必要がある。

有害性発現経路(AOP)の開発

主要情報部分の特定

17. AOPの基本的な構成要素は、通常、生物学的組織の異なるレベルにあるKEsである。これらのKEは、検討中のAOに因果関係があり、不可欠であり、測定可能である。MIE と AO は、単一のAOPの状況下において、特殊なKEsを必要とする。AOPは生物学的標的との化学物質の直接的な相互作用を表すMIEによって一方の端に固定され、もう一方の端には規制上の決定に関連する生物学的レベルの組織であるAOで固定されている。AOP の 2 番目の基本構成物は、主要事象の 関係 (KER) です。KER はAOP の上流の KE と下流の KE の間をつなぐ。これらの情報は、AOP開発時に明確に識別し、記述する必要がある。

18. AOPを開発するために、経路(すなわち経路を開始する分子標的と相互作用する可能性を持つ)を開始できる化学物質の種類を反映する構造アラート、相対反応性または化学的-生物学的相互作用を測定する*in chemico*法、一連の細胞応答(例えば遺伝子発現)を与える*in virto*法、*ex vivo*および*in vivo*機構試験、直接指標(複数可)を測定する*in vivo*試験において、規制上の意思決定を推進するAOに関連する指標を測定する*in vivo*試験を含むさまざまな種類のデータを利用することができる(OECD 2011,ENV/JM(2011)6)。AOPは化学的に特異的ではなく、記述経路は特定の化学初動からの独立を繰り返していることに価値がある。それにもかかわらず、特定のAOの状況下において、化学物質への曝露から得られた実験データは、生物学的応答の様式を理解するために有用である。この情報は、AOP 内の KE および KE を識別し、AOP を裏付ける科学的証拠を提供するために使用される。したがって、AOPは、異なる次元(例えば、異なるレベルの生物学的組織)における効果をAOPの最終指標に結びつける科学的基礎を提供する(図1)。

分子初動事象の定義(作用部位)

19.化学的に誘導された生物学的システムの摂動は、分子レベルに始まる。ほとんどの化学物質は、複数の分子標的と相互作用する。MIEは、AOPの主要なアンカーまたは「基盤」である。したがって、特に、MIE/AOPが*in silico*または*in chemico*予測を支援するために使用されている場合は、評価に関連する特定の最終AOにつながる始まりを明確に識別することが重要である。多くのMIEは、タンパク質および/またはDNAに対する共有結合の形で定義されている。これらのタイプのMIEは、有機化学（すなわち求電気基球反応性）の原理に基づいている。対照的に、「受容体結合」または酵素への結合は、多くの場合、自然界でより選択的である非共有結合相互作用に基づいている。化学物質は、異なるターゲットに対して異なる親和性を持っている。内部曝露が受容体または酵素上の結合部位を飽和するのに十分であるならば、活性の活性化または阻害の効力は毒性を引き起こすかもしれない。MIEの理解は、生物学的利用可能性、構造要件（特に受容体結合）および代謝変化などの摂動を誘発する可能性が最も高い化学物質の特性の同定と定義の理解を容易にする。潜在的な化学的誘導物質を理解することは、同様の方法で作用する化学分類物質の分子構造の限界を定義するのに役立つ。

20.理想的なシナリオでは、MIEが明確に定義されている場合、その事象を引き出す化学物質の可能性が認識されるだけでなく、摂動の活性部位にも注目すべきである。例えば、求電子性の種に対する物質の代謝変換は、皮膚感作および肝線維症に関して同じであってもよいが、作用部位は異なることになる(ケラチノサイト対肝細胞)。いくつかの指標、特に受容体結合機構に基づいての作用部位の同定は、受容体の「立体構造」および他の特性が構造的にそれに結合できる分子の種類を定義するので非常に重要である。

しかし、MIEの作用の部位の同定が非常に困難であるかもしれない（例えば、反復投与）または正確に定義されていない（例えば、魚の単純ナルコシス）AOが多数存在する。 定義されてい

ない機能部位を持つAOPは有用ではなく、必要ではない。

有害発現の特定

21. AOは、一般的に、承認された規制ガイドライン毒性試験での確立された保護目標または先端指標に対応する規制的に重要性であるとして承認されている専門的なタイプの主要事象である(OECD、2016a)。AO は、さまざまな次元 (例えば、曝露の　持続時間、性別、種など)に基づいて定義できる。最終的なAOは、個人レベルまたは集団レベルにおける特定のAOPのアンカーである。規制上の意思決定に関連する最終的なAOを明確かつ正しく定義することが不可欠である（すなわち、それは確立された規制ガイダンスの承認された保護目標または共通の先端指標に対応する)。これは、ヒト健康の場合、個人または集団全体または特定された集団のいずれかに、特定の臓器または器官系における病状のリスクの増加を促すAOにつながる事象の機械学的連続を説明するのに役立つ。

一方、生態系では、野生動物の集団持続可能性の推定値の観点から意味を持つ集団統計学的意義の結果であることが最も多い。生存、胎児性または成長に関連した結果は、容易に集団の持続可能性に関係する。しかし、生物の多くの構造的（例えば、重大な異常）および機能的（例えば、行動異常）な変化は、他の要因と組み合わせると調節的意義を有し、ヒトおよび生態学的リスク評価における貴重な証拠を付加する可能性がある。少なくとも、AOPは、組織の器官レベル以上で規制上重要な少なくとも一つのAOに関連する必要がある。可能な場合は、ヒトの健康と生態学的リスク評価のためのAOPの有用性を最大化するために、KERの連続を集団レベルまで拡張する必要がある（適用範囲内にて）。

有害経路につながる主要事象の特定

22. KEは、通常、さまざまなレベルの生物学的組織で、中間事象経路に沿った段階として定義される。KEになるためには、AOPの中間段階にあることが不可欠であり、実験的に測定可能でなければならない。 ユーザーハンドブックには、AOPにKEを含めるために使用できるデータの種類に関する追加の考慮事項と事例が記載されている。

23. AOPには、評価に関連する最終AOとMIEの間にあるKEの収集が含まれる。隣接する事象間の関係は、多くの場合、すべての中間事象を記述するのではなく、経路に沿って発生する主な効果をとらえ、重要で測定可能なKEを含めることができる方法と定義できる。 理想的なシナリオでは、AOPには、MIE（アンカー1）と最終AO（アンカー2）の因果関係/接続を確立するために必要な比較的少数または最小限のKEを含める必要がある。 AOPを評価するための支援とデータを提供するため、KEおよびKERに関するさまざまな*in vivo*および*in vitro*の情報、およびハイスループットスクリーニング（HTS）アッセイからの情報、ハイコンテンツスクリーニング（HCS）からの指標、オミクスアプローチさらに、*in silico*法も使用できる。 KEの数が増えると、毒物学的な複雑さが明らかになる。

24. AOにつながるKEを特定する前に、正常な生理学的経路の理解が不可欠である（例：生殖段階、肝機能）。これは、生物学的組織のさまざまなレベルでかく乱する可能性のある過程の複雑なネットワークの認識に役立つ。 KEの特定中に、既存の文献のレビューは、最終的な悪影響につながる妥当な機構と中間段階について可能な限り多くの情報を評価するために必要である。入手可能な文献のKEデータの信頼性と関連性を判断するには、最終AOに関する比較と解釈のために、研究計画の重要な因子（曝露摂生、曝露期間、サンプリング時間）の評価が含まれる。系統的**レビュー**を含む自動化された文献を掘り起こし、評価ツールは、AOPの開発を加速し、データ評価の客観性と透明性を高めるのに役立つが、必須ではない。重要な点は、AOPの開発は科学文献によって支援されるべきであり、それがどのように達成されるかは、ユーザーズハンドブック

で議論されている主要な考慮事項が扱われる限り、AOPの開発者次第である。通常、複数の中間事象は、特定のAOPの構築中に同定される。 したがって、集められた知識は、関心のある特定のAOPに適切であるというフィルターをかける必要がある。 KEが複数のAOPに存在する場合、情報はAOP間で共有できる。

主要事象関係の特定

25. AOPで識別されたすべてのKEは関連し、KERで定義される。 AOPでのKERの記述には、上流のKEの既知の状態または測定された状態から下流のKEの推定変化または状態を推定するための科学的根拠を定義する情報と証拠の種類を集めて整理することが含まれる。

26. 慣例により、KERは2つの形式のいずれかをとることができる（OECD、2016a）。 KERを介してリンクされたKEのペアは特定のAOPを定義するKEの連続で互いに隣接している場合がある。あるいは、KERは、関係が別のKE（つまり、AOP内の非隣接KE）を通過すると考えられるKEのペアを指す場合がある。隣接しない可能性のあるKEの可能な２つ毎にKERを記述する必要はない。ただし、隣接していないKEのKER記述を提供するオプションは、特にAOP Wiki内で役立つ。隣接するKE間の関連を支援する経験的証拠が利用できないか、直接隣接していないKEのみが利用できるためである。 たとえば、一部のKEの測定は、通常の研究ではめったに行われないように、行うのがかなり難しい場合がある。 AOPの一部としてKEを確立するのに十分なデータがあるかもしれないが、利用可能なWoEの多くは、その特定のKEを無視するか、「飛び越える」ことができる。隣接しないKEの記述を含めると、これらの関係のWoEを記述し、他のAOPに連結できる。

27. AOP開発の重要な要素は、KERのWoEの評価である。これには、関係の生物学的妥当性の評価、および最初のKEが後続のKE以下の用量で、下流のKEよりも早い時点で発生することを裏付ける経験的証拠の検査が含まれる。事例などの詳細情報は、以下およびユーザーズハンドブックの補遺にある。

AOP評価

28. 規制目的での使用を意図して開発された特定のAOPの基礎となる証拠は、MOA背景でのWoEの比較分析に使用される進化したBradford-Hillの考慮事項に基づいて評価される（Meek et al., 2014a; Meek et al., 2014b）。 ただし、必要に応じて、化学的にとらわれないAOPの状況に対応するように変更されており、ユーザーズハンドブック（OECD、2016a）に記載されている。

29. 3つの主要な考慮事項は次の通り

1) KERの生物学的妥当性。これは、関与する基本的な生物学的段階と、それらがAOPで提案されている因果関係と一致しているかどうかの理解に依存している。
2) KEの本質。AOP全体の状況下で考慮され、上流のKEがブロックされた場合に下流のKEまたはAOが防止または変更されるかの実験データを指す（ノックアウトモデルでの試験または 可逆性）
3) KERの経験的支援。これは、多くの場合、KEペアの用量反応と時間的一致を評価できる一津つ以上の参照化学物質から得られた毒性データに基づいている。

30. AOPを評価する場合、生物学的妥当性と経験的支援の両方がKER毎に個別に評価されるが、KEのそれぞれの支援情報に基づいて、AOPの状況下では本質性が考慮される。 その段階は、前述のようにAOP間の構成物の共有を支援するだけでなく、AOP内の重要なデータのギャップと不確実性を明確に特定し、規制活用に目標を絞った研究と評価を促進するのに役立つ。 WoE（生物学的妥当性と経験的支援で構成される）が各KERで評価され、各KEで本質性が評価されると、

AOP全体を支援する証拠をユーザーハンドブック（OECD,2016a）の指示に従って表にまとめることができる。

AOPの利用

31. リスク評価の基礎として有害性の定量化を支援するには、数学的にモデル化できる記述的な定量的KERを備えた、よく記述説明されたAOPが通常必要である。 しかし、明確に定義された定性AOPでさえ、さまざまなレベルの生物学的組織を通じて正確に記述された一連の事象で、多くの目的に使用できる貴重な機構情報を提供できる（OECD 2011、ENV / JM（2011）6）。

32. AOPのさまざまな潜在的な用途が説明されている。 特定のAOPの決定を支援できる範囲は、不確実性のレベルとKERの定量的理解に依存する。たとえば、KEを特定して説明することにより、AOPは特定の方法における使用の理論的根拠を説明し、潜在的により予測的な方法の開発により、OECD試験法ガイドラインプログラムの作業に通じる（以下でさらに説明）。 AOPは、IATAまたは統合的試験戦略（ITS）を開発するための基礎としても使用できる。 また、区分を最初に形成し、区分内でデータギャップを埋めるRead Acrosssなど、代替アプローチのさらなる開発と適用にも使用できる。これにより、従来の生体内試験の改良、削減、および/または置換が可能になる。

33. AOPは、以下に限定されない多くの規制状況に貢献するために使用できる：そのため、（1）から（4）に進むと、許容できる不確実性のレベルが低下し、AOP開発を支援する際に提示される証拠のレベル（詳細、品質、および情報とデータの量など）が増加する。

34. 部分的に開発されたAOP（つまり、すべてのKEが知られているわけではないもの）は、さらなる試験と開発の優先順位設定に役立つ場合がある。 同様に、現在OECD QSAR Toolboxで実行されているように、部分的に開発されたAOPが有害性識別に使用される場合がある。 生理学に基づいた薬物動態（PBPK）モデリングおよび吸収、分布、代謝、排泄（ADME）に関するトキシコキネティクス情報は、AOP開発では考慮されないが、上記の規制状況のいずれかでAOPの適用に対処する必要がある。 AOPは、特定の化学物質のMOA分析の開始点としても機能し、化合物空間とADMEの考慮を取り入れることができる。

35. 定性的AOPは、AOPを支援するWoE全体の定性的評価、妥当性または統計的推論に加えて、KEの測定方法の説明およびKEが経験的証拠によって支援される記述によってKEが支援される（Villeneuve et al., 2014a）。 対照的に、定量的AOPは、KEの測定方法の説明によって支援されるKEの機械言語と、変化の大きさおよび/または持続時間の定量的理解によって支援されるKERとともに測定が行われる精度と精度に基づいている 下流のKEにある程度の変化を引き起こすには、上流のKEが必要である（Villeneuve et al., 2014a）。

36. AOPはその開発中に、推定AOPとして始まり、より多くの情報が蓄積されるにつれて定量的AOPに進化する可能性がある。一般に、AOPは推定、完全定性、完全定量の間の連続体に沿って存在する。 AOPは決して完全ではないことに注意することが重要である。AOPは進化し続けることができるが、意思決定を支援するためにその使用を妨げるものではない。 OECD内でのAOPの潜在的な用途を以下に説明する。

化学品区分の開発とOECD QSARツールボックスのさらなる開発

37. AOPの主な対象活用の一つは、Read acrossおよび化学物質区分形成の分野である。 AOPは、一つまたは複数のAOP内のMIEとKEの類似性に対処することにより、OECD QSAR Toolboxを使用する場合、グループ化アプローチにおける物質の生物学的類似性に関する作用機構情報を提供

できる。 さらに、AOPは、特定のKEの試験に焦点を当てることにより、化学物質区分内の洗練された試験法戦略の識別を支援し、構造的に類似した物質の共通の作用モードに対処できる（試験と評価のための統合アプローチの状況下でのAOPの使用も参照）。

38. 実証されているように、OECD QSAR Toolboxのバージョン3.0で皮膚感作を引き起こすタンパク質結合については、AOPを使用して化学物質区分を開発および改良できる。 この例では、3つの情報セットが照合および統合される。（1）評価で通常使用される生体内効果のライブラリ（例、局所リンパ節試験のEC3値）、（2）MIEのライブラリ（例、タンパク質結合反応）、および（3）中間事象のライブラリ、通常は*in vitro*法を使用して作製されたデータ（樹状細胞表面バイオマーカーなど）（OECD 2012, ENV / JM / MONO（2012）10 / PART1; ENV / JM / MONO（2012） 10 / PART2）。 理論的には、各指標は単一または複数の化学物質の範囲内に関連付けることができる。 化学物質の区分に関しては、対象となる化学構造空間、または適用領域は、AOP内のMIEおよびKEに対して評価される化学物質に依存する。

試験法ガイドラインプログラム

39. KEを特定して説明することにより、AOPは試験法ガイドラインプログラムの作業に通じる。 実際、KEが特定されると、細胞またはより高いレベルの生物学的組織で直接的な化学効果または応答を検出する*in vitro*および*ex vivo*試験法の開発、および特定されたMIEに関連する目的のスクリーニング試験の開発を提案できる（ OECD 2011, ENV / JM / MONO（2011）8）。 逆に、*in vitro*試験法の開発に関する提案をAOPのKEに連結することにより、規制目的に関連する有害性指標との関係を確立できる。

40. たとえば、皮膚感作をもたらすタンパク質結合についてAOPで特定された2つの方法が、試験法ガイドラインの開発のためにOECDに提案されている。ケラチノセンスアッセイ（ヒトケラチノサイトでの遺伝子発現）（OECD、TG No.442CおよびD）および h-CLATアッセイ（ヒト単球細胞における細胞表面マーカー(CD86) 発現）（OECD、TG No. 442E）である。

41. しかし、単一のAOPにより規制に関連する可能性のあるすべての事象を捉えることはほとんどない。 少なくとも一つの共通要素を共有するAOPに基づくAOPネットワークは、潜在的にAOに至る経路とネットワークの変更より現実的になる。 これらのAOPネットワークの分析は、複数の結果の予測実用性を備えた単一の試験法を開発することであろうと、規制上の懸念に関する特定の指標を予測するために非常に特異的な一連の試験法を開発することであろうと、試験法開発の優先順位付けに役立つ。 たとえば、魚の生殖および発生毒性に関連する5つのAOPを使用して、AOPネットワークを記述し、AOPネットワークを試験法の開発と改良に使用する方法を説明している(Knapen et al., 2015)。

試験法と評価のための統合アプローチ（IATA）の状況下でのAOPの使用

42. AOPは、特定の指標のIATAまたはITSを開発するための基盤として機能する。 AOPは、どの追加情報（および、もしあれば、どの試験）が初動事象と悪影響に関連する確実性を高めるかを決定するのに役立つ。さらに、確立されたAOPを使用して、種から種への外挿に使用できる。 AOPの基礎となる信頼性と信頼性、およびIATAでのそれらの適用が提案されている(Tollefsen et al., 2014; Patlewicz et al., 2015; Perkins et al., 2015)。 IATAの開発と使用のための枠組みに関する2014年に開催されたワークショップ（OECD, 2015年）に基づいて、IATAの開発におけるAOP概念の使用のための手法の概要を記したガイダンスが利用可能である。

要約

43. リスク評価の予測戦略を実施するには、MIEまたはMIEに対する細胞応答に焦点を合わせた*in vitro*毒性試験の結果を、生物および最終的には集団への影響に外挿する必要がある。 これは、MIEとAOを因果的に関係するAOPの開発により実現できる。 これらのAOPは規制当局による使用を目的としているため、このガイダンスでは、AOPの開発、評価、およびAOPの開発、文書化、レビューの方法の標準化を目的としている。 承認されたAOPは、その開発とレビューに続いて、AOPに関するOECDシリーズで公開されている。 ただし、科学的知識が進歩するため、このシリーズのAOPの公開は、そのAOPへのさらなる更新または新しい貢献を妨げるものではない。

44. AOPは、化学的有害性分類を開発または改良するための、透明性があり、作用機構に基づいた枠組みを提供し、目的となる*in vitro*および*in vivo*試験の提案および優先順位付けする必要がある。 構造活性相関（SAR）および*in chemico*および*in vitro*試験から、より低いレベルの生物学的組織における影響の可能性を理解することにより、より高いレベルの生物学的組織（たとえば*in vivo*試験）で追加の試験が必要かどうかを効率的に判断できる（ Meek et al., 2011）。 ここで提供されるガイダンスは、MOA枠組み（2012年にWHO / IPCSにより更新; Meek et al., 2014a）で提示され、ユーザーズハンドブックに記載された進化するMOA分析の組み込みとともに、意思決定プロセスにおける機械的データと計算モデルの組み込みを支援する。

45. Bauchらにより示されているように、AOP内のすべてのKEが、評価で使用されるために完全に記述されている必要はない（Bauch et al., 2011)。特定の目的のためのAOP またはAOPネットワークの使用には、 各KERおよび 全体的なAOPと同様に、各KERの評価の基礎である最終AOにつながるMIEおよびKEに関する情報の結果を含んでいる。AOPの十分な知識と考えられるものは用途に依存する可能性があり、潜在的な影響が大きな応用により知識や信頼度が高くなる（Meek et al.,2011）

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補遺 I: 有害性発現経路に関する用語集

このガイダンス文書に記載されている用語は、アルファベット順に整理されている。 以下の用語のいくつかは、定義が大きく重複しているさまざまなソースで説明されている。 ただし、これらの用語の最も進化した完全な定義は、考慮のためにここに含まれている。

ADME
薬物動態/毒物動態学および薬理学/毒物学で用いられる吸収（Absorption）、分布（Distribution）、代謝（Metabolism）、排泄（Excretion）の頭字語。生物内の医薬品/化学化合物の性質を示す。 4つの段階はすべて、薬物/化学物質のレベルと組織への薬物曝露の動態に影響を与えるため、化合物の性能と薬理/毒物学的活性に影響する（Pharmacology Study Guide、2007）。

Adverse outcome
有害作用は、確立された保護目標への対応または承認された規制ガイドライン毒性試験（OECD、2016a）の先端指標との同等性に基づいて規制上の重要性があるとして一般に受け入れられている特殊なタイプの主要事象である。

注：保護の目標が人間の健康か環境の健康かによって、考慮される指標は異なる場合がある。

Adverse Outcome Pathway (AOP)

概念的には、AOPは、ストレッサーと標的細胞または組織内の生体分子との最初の相互作用から始まる一連の事象（つまり、分子開始事象）として見ることができ、一連の依存事象を経て進行し、有害な結果に至る代償作用とフィードバックループが克復されると、AOPは通常、1つの重要な事象から別の事象に移動して順番に表される (OECD, 2016a)。

Apical endpoint
先端指標は、死亡、発達異常、繁殖行動、生殖障害、身体的変化、および疾患状態を示す臨床徴候または病理学的状態を含む臓器の大きさおよび組織病理学の変化など、曝露の経験的に検証可能な結果である (Krewski et al., 2011; Villeneuve and Garcia-Reyero, 2011)。

注：先端とみなされる指標（結果）は、人間の健康と生態学的な健康の代理として使用される場合、異なる場合がある。

Cellular response

化学物質の対応する受容体への結合は、細胞内で最終的にその攪乱を変化させる事象を誘発する。これらの細胞内事象の性質は、受容体の種類によって異なる。 また、同じ化学シグナルが異なる細胞タイプで異なる反応を引き起こす可能性がある (http://global.britannica.com/EBchecked/topic/101396/cell/37445/Cellular-response).

Chemical category

物理化学的および人間の健康および/または環境毒物学的特性および/または環境運命特性が類似している可能性が高い、または構造的類似性（または他の類似性特性）の結果として規則的なパターンに従う化学物質のグループ (OECD, 2007).

Effectopedia

Effectopediaは、AOP開発、モデリング、使用のための共同研究プラットフォームである。知識（定性的および定量的）は入れ子になった層で構成される。経路表現の最も抽象的な層は、経路構造の視覚的な図である。次のレベルの詳細は、個々の要素（化学物質、効果、リンク、試験法、in silicoモデルなど）のカスタムビルドインターフェイスで取り込まれ、構造化された要約情報の統一表現を提供する。Effectopediaの全体的な目標は、定量的AOPの開発と規制上の意思決定の状況（Aladjov、パーソナルコミュニケーション）での使用を可能にするために必要なすべての情報を単一源に集約することである。

Endpoint

*in chemico*法、*in vitro*試験法または*in vivo*試験から得られ、記録された結果(OECD, 2011).

Integrated Approaches to Testing and Assessment (IATA)

試験法と評価への統合アプローチは、化学物質の区分識別、有害性評価、および/または安全性評価に使用される複数の情報源に基づく手法である。IATAは、関連するすべての既存の証拠を統合および重み付けし、必要に応じて、潜在的な有害性および/またはリスクに関する規制上の意思決定を通知するために、新しいデータの目的形成を説明する。 IATA内では、さまざまな情報源（物理化学的特性、*in siloco*モデル、グループ化およびRead Across アプローチ、*in vitro*試験法、*in vivo*試験、およびヒトデータ）からのデータが評価および統合され、有害性および/またはリスクに関する結論が導き出される。この段階内で、非動物試験および非試験方法で生成されたデータの組み込みは、動物での試験の削減に大きく貢献すると予想される（OECD、2016b）。 IATAの出力は、他の考慮事項とともに、規制上の意思決定に役立つ結論である (OECD, 2016b)。

Integrated Testing Strategy (ITS)

試験および評価に対する定義済みの手法はさまざまな方法で設計でき、たとえば、連続試験戦略（STS）または統合的試験戦略（ITS）の形式をとることができる。ITSはさまざまな特定の方法論を適用して、異なる情報源からの入力を予測に変換することにより、複数のデータまたは情報源を同時に評価する手法である。この目的のために、統計モデルや数学モデルなど、さまざまな特定の方法論を適用できる (OECD, 2016b)。

Key Event (KE)

主要事象は、特定の有害な結果につながる定義済みの生物学的摂動の進行に測定可能で不可欠な生物学的状態の変化(OECD, 2016a)。

Key event relationship (KER)

主要事象の関係は、一つの主要事象を別の主要事象に接続し、二つの間の有用な関係を定義し（つまり、一方を上流として、もう一方を下流として識別）、上流に主要事象の既知、測定、あるいは予測状態から下流の状態の推論または外挿を容易にする科学的根拠の関係（OECD, 2016a)。

Levels of biological organisation

原子、分子、細胞、組織、器官、器官系、生物（個体）、集団、コミュニティ(図１参照)

(Villeneuve and Garcia-Reyero, 2011)。

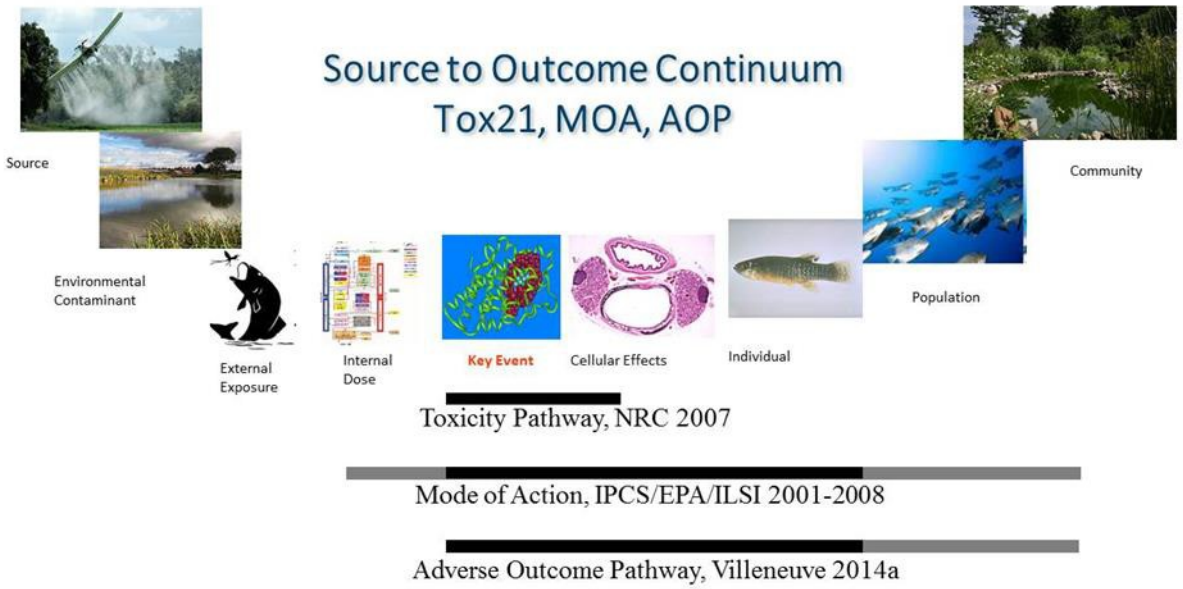

図1. 毒性経路、作用機序経路および有害転帰経路の間の関係の表現。黒いバーはこれらの概念に共通する幅広い研究を表す。 灰色のバーは、概念の理論的な範囲を表す (adapted from OECD 2011).

Mechanism of action

毒性の作用機序は、がんまたはその他の健康指標の誘発における重要な事象の詳細な分子記述である。作用機構は、作用形態が意味するものよりも詳細な事象の理解と説明を表す(North American Free Trade Agreement NAFTA, 2011)。

Mode of action (MOA)

作用機序は、WHOによって「生物学的にもっともらしいKEの連続として定義されており、粗い実験的観測と機械的データによって裏付けられた観測された効果をもたらす。作用形態は、重要な細胞学的および生化学的事象、つまり、測定可能であり、観測された効果に必要な事象を論理的な枠組みで説明する。 World Health Organization (2009) Environmental Health Criteria 240: Principles and Methods for the Risk Assessment of Chemicals in Food. WHO, Geneva, (Definitions page A-25) http://www.who.int/foodsafety/publications/chemical-food/en/.

Molecular Initiating Event (MIE)

分子開始事象は、AOPを開始する攪乱をもたらす生物内の分子レベルでの化学的相互作用の初期点を表す特殊なタイプの主要事象である (OECD, 2016a)。

Molecular screening

分子スクリーニングは、迅速なスクリーニング法とトキシコゲノミクスを、生化学および細胞ゲノム法をカテゴリー分析に適用する目的と組み合わせている。分子毒性スクリーニングの前提は、化学物質と何らかの形の細胞標的との相互作用によって引き起こされる。最初に毒性を評価するには、適切な懸念対象を特定する必要があり、懸念の化学物質と相互作用の可能性を評価するには適切な試験が必要である。 (OECD, 2008).

Pathway perturbation

有害な健康影響が発生する程度まで正常な生物学的機能を損なう可能性のある環境因子またはその代謝物による毒性経路の重大な変化(Krewski et al., 2011).

Site of action

作用部位は、化学物質と相互作用する生体分子であるか、受容体のリガンド結合ドメインなど、目的の高分子上のより特異的な部位を指す。作用部位は、分子開始事象が発生する特定の細胞または組織タイプの状況と見ることもできる（シュルツ、個人的なコミュニケーション）。 作用部位は種固有である場合があることに注意。

Structural alerts

構造アラートは、原子ベースのフラグメントであり、分子内に存在する場合、化合物を特定のカテゴリーに配置できることを示す(Schultz, 2010)。

Systems biology

システム生物学は、動的に相互作用するネットワークの生物学として定義される。それは、生の全体を形成するネットワークはその部分の合計以上のものであるという理解から始めて、生物システムの複雑さを解読することを目的とした生物医学の全体論的アプローチである。Systems biologyは、生物学、コンピューターサイエンス、エンジニアリング、バイオインフォマティクス、物理学、およびその他の分野のアプローチを統合し、これらのシステムとネットワークが化学的曝露を含むさまざまな条件下でどのように変化するかを予測する。バイオインフォマティクス、データ統合、モデリングに加えて、さまざまな分析プラットフォームの使用が必要である(Jain, 2010)。

注：システム生物学には、（1）大量の実験データの収集（ハイスループット技術および/または還元主義分子生物学および生化学の文献のマイニングによる）、（2）少なくともいくつかの重要な側面を説明する可能性のある数学モデルの提案が含まれる。このデータセットの（3）数値予測を得るための数学方程式の正確なコンピューターソリューション、および（4）数値シミュレーションと実験データの比較によるモデルの品質の評価 (Duffus et al., 2007).

Toxicity Pathway

十分に摂動すると、健康への悪影響をもたらすと予想される細胞応答経路は、毒性経路と呼ばれる（NRC、2007）（図1内で定義）。 毒性経路は、分子開始事象から細胞効果への正常な生化学経路の摂動に関連している。 MOAとAOP概念の中心にあるが、先端効果に直接リンクしていない。

Weight of evidence (WoE)

WoEは、状況に応じてアプローチとツールが異なる仮説を裏付ける情報の範囲と質の、包括的で統合された、しばしば定性的な判断である（Weed、2005; WHO-UNEP、2012）。AOPの場合、疫学研究（Hill、1965）で因果関係を評価するためにBradford Hill（B / H）によって提案されたものから修正された特定の考慮事項のサブセットに基づいて、WoEが対処される。関連する各考慮事項の質問と補助データの性質の定義は、AOPに関するOECDガイダンスのユーザーハン度ブックの補遺 1 に含まれる (OECD, 2016a)。

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Section 4
Health effects

Test Guideline No. 442C

In Chemico Skin Sensitisation

Assays addressing the Adverse Outcome Pathway key event on covalent binding to proteins

18 June 2019

OECD Guidelines for the Testing of Chemicals

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Key–Event-Based Test Guideline For In Chemico Skin Sensitisation Assays Addressing The Adverse Outcome Pathway Key Event On Covalent Binding To Proteins

GENERAL INTRODUCTION

Covalent binding to proteins Key Event based Test Guideline.

1. A skin sensitiser refers to a substance that will lead to an allergic response following repeated skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). There is general agreement on the key biological events underlying skin sensitisation. The current knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised as an Adverse Outcome Pathway (AOP) (2) starting with a molecular initiating event through intermediate events to the adverse effect, namely allergic contact dermatitis. This AOP focuses on chemicals that react with amino-acid residues (i.e. cysteine or lysine) such as organic chemicals. In this instance, the molecular initiating event (i.e. the first key event), is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The second key event in this AOP takes place in the keratinocytes and includes inflammatory responses as well as changes in gene expression associated with specific cell signaling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways. The third key event is the activation of dendritic cells, typically assessed by expression of specific cell surface markers, chemokines and cytokines. The fourth key event is T-cell proliferation.

2. The assessment of skin sensitisation has typically involved the use of laboratory animals. The classical methods that use guinea-pigs, the Guinea Pig Maximisation Test (GPMT) of Magnusson and Kligman and the Buehler Test (OECD TG 406) (11) assess both the induction and elicitation phases of skin sensitisation. The murine tests, such as the LLNA (OECD TG 429) (12) and its three non-radioactive modifications — LLNA:DA (OECD TG 442A) (13), LLNA:BrdU-ELISA, and BrdU-FCM (OECD TG 442B) (14) — all assess the induction response exclusively and have gained acceptance, since they provide an advantage over the guinea pig tests in terms of animal welfare together with an objective measurement of the induction phase of skin sensitisation.

3. Mechanistically-based in chemico and in vitro test methods addressing the first three key events of the skin sensitisation AOP have been adopted for contributing to the evaluation of the skin sensitisation hazard potential of chemicals: the present Test Guideline assesses covalent binding to proteins, addressing the first key event; the OECD TG 442D assesses keratinocyte activation (15), the second key event and the OECD TG 442E addresses the activation of dendritic cells (16), the third key event of the skin sensitisation AOP. Finally, the fourth key event representing T-cell proliferation is indirectly assessed in the murine Local Lymph Node Assay (LLNA) (12).

Background and principles of the test methods included in the Key Event based Test Guideline

4. This Test Guideline (TG) describes in chemico assays that address mechanisms described under the first key event of the AOP for skin sensitisation, namely covalent binding to proteins (2). The Test Guideline comprises test methods to be used for supporting the discrimination between skin sensitisers and non-sensitisers in accordance with the UN GHS (1). The test methods currently described in this Test Guideline are:

- The Direct Peptide Reactivity Assay (DPRA) (Appendix I), and
- The Amino acid Derivative Reactivity Assay (ADRA) (Appendix II).

5. These two test methods are based on in chemico covalent binding to proteins and are considered to be scientifically valid. The DPRA has been evaluated in a European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)-lead validation study and subsequent independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC) (3) (4) (5). The ADRA underwent a validation study coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM) (6) (7) (8) (9) followed by an independent peer-review (10).

6. The test methods included in this Test Guideline might differ with regard to the procedures used to generate the data but can each be used to address countries' requirements for test results on protein reactivity, while benefiting from the Mutual Acceptance of Data.

7. The correlation of protein reactivity with skin sensitisation potential is well established (17) (18) (19). Nevertheless, since protein reactivity represents only one key event of the skin sensitisation AOP (2) (20), information generated with test methods developed to address this specific key event may not be sufficient as stand-alone methods to conclude on the presence or absence of skin sensitisation potential of chemicals. Therefore data generated with the test methods described in this Test Guideline are proposed to support the discrimination between skin sensitisers (i.e. UN GHS Category 1) and non-sensitisers when used within Integrated Approaches to Testing and Assessment (IATA), together with other relevant complementary information from in vitro assays addressing other key events of the skin sensitisation AOP as well as non-testing methods, including in silico modeling and read-across from chemical analogues (20). Examples on the use of data generated with these methods within Defined Approaches (DAs) i.e. approaches standardised both in relation to the set of information sources used and in the procedure applied to derive predictions—have been published (20) and can be employed as useful elements within IATA.

8. The test methods described in this Test Guideline do not allow either sub-categorisation of skin sensitisers into subcategories 1A and 1B (21), as defined by UN GHS (1) for authorities implementing these two optional subcategories, or potency prediction for safety assessment decisions. However, depending on the regulatory framework, positive results generated with these methods may be used on their own to classify a chemical into UN GHS Category 1.

9. Definitions are provided in the Annex. Performance Standards for the assessment of proposed similar or modified in vitro skin sensitisation DPRA and ADRA test methods have been developed (22).

Literature for introduction

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ANNEX - DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with concordance to mean the proportion of correct outcomes of a test method (1).

(Formula shown below.)

ADRA: Amino acid Derivative Reactivity Assay

AOP (Adverse Outcome Pathway): sequence of events from the chemical structure of a target chemical or group of similar chemicals through the molecular initiating event to an in vivo outcome of interest (2).

Calculation

Calculating depletion of either NAC or NAL

Depletion is calculated as follows:

Percent depletion of either NAC or NAL = {1- (NAC or NAL peak area in replicate injection ÷ mean NAC or NAL peak area in reference control C)} × 100

Calculating predictive capacity

There are several terms that are commonly used along with the description of sensitivity, specificity and accuracy. They are true positive (TP), true negative (TN), false negative (FN), and false positive (FP).

Sensitivity, specificity and accuracy are described in terms of TP, TN, FN, and FP.

Sensitivity: Number of true positives ÷ Number of all positive chemicals, TP ÷ (TP + FN)

Specificity: Number of true negatives ÷ Number of all negative chemicals, TN ÷ (TN + FP)

Accuracy: Number of correct predictions ÷ Number of all predictions, (TN + TP) ÷ (TN+TP+FN+FP)

Calibration curve: The relationship between the experimental response value and the analytical concentration (also called standard curve) of a known substance.

Coefficient of variation: a measure of variability that is calculated for a group of replicate data by dividing the standard deviation by the mean. It can be multiplied by 100 for expression as a percentage.

Defined Approach (DA): a DA consists of a fixed data interpretation procedure (e.g. statistical, mathematical models) applied to data (e.g. in silico predictions, in chemico, in vitro data) generated with a defined set of information sources to derive a prediction.

DPRA: Direct Peptide Reactivity Assay

EDTA: Ethylenediaminetetraacetic acid

EURL ECVAM: the European Union Reference Laboratory for Alternatives to Animal Testing

Hazard: Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

IATA (Integrated Approach to Testing and Assessment): A structured approach used for hazard identification (potential), hazard characterisation (potency), and/or safety assessment (potential/potency and exposure) of a chemical or group of chemicals, which strategically integrates and weights all relevant data to inform regulatory decision regarding potential hazards, risks, and the need for further targeted and therefore minimal testing.

JaCVAM: Japanese Center for the Validation of Alternative Methods

LLNA: murine Local Lymph Node Assay issued as OECD TG 429 in 2010

Molecular Initiating Event: Chemical-induced perturbation of a biological system at the molecular level identified to be the starting event in the adverse outcome pathway.

Mixture: A solid or liquid comprising two or more substances which do not react chemically. (3)

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent comprises at least 80% (w/w) of the whole.

Multi-constituent substance: A substance, defined by its quantitative composition, in which two or more main constituents are present in concentrations ≥ 10% (w/w) and < 80% (w/w). Multi-constituent substances are the result of a manufacturing process. The difference between a mixture and a multi-constituent substance is that a mixture comprises two or more substances which do not react chemically, whereas a multi-constituent substance comprises two or more substances that do react chemically.

NAC: N-(2-(1-naphthyl)acetyl)-L-cysteine (4) (5) (6)

NAL: α-N-(2-(1-naphthyl)acetyl)-L-lysine (4) (5) (6)

Positive control: A replicate containing all components of a test system and treated with a substance known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Pre-haptens: chemicals which become sensitisers through abiotic transformation

Pro-haptens: chemicals requiring enzymatic activation to exert skin sensitisation potential

Reference control: An untreated sample containing all components of a test system, including the solvent or vehicle that is processed with the test chemical treated and other control samples to establish the baseline response for the samples treated with the test chemical dissolved in the same solvent or vehicle. When tested with a concurrent negative control, this sample also demonstrates whether the solvent or vehicle interacts with the test system.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method. (1)

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability. (1)

Reproducibility: The concordance of results obtained from testing the same substance using the same test protocol (see reliability). (1)

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method. (1) (Formula shown below.)

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method. (1) (Formula shown below.)

Substance: Chemical elements and their compounds in the natural state or resulting from a manufacturing process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process, but excluding solvents that may be separated without affecting the stability of the substance or changing its composition (3).

System suitability: Determination of instrument performance (e.g. sensitivity) by analysis of a reference standard prior to running the analytical batch (7).

Test chemical: The term test chemical is used to refer to the substance being tested.

TFA: Trifluoroacetic acid

United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to standardised types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (3).

UVCB: substances of unknown or variable composition, complex reaction products or biological materials.

Valid test method: A test method considered to have sufficient relevance and reliability for a specific purpose and which is based on scientifically sound principles. A test method is never valid in an absolute sense, but only in relation to a defined purpose (1).

Literature for definitions

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APPENDIX I

In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

10. The DPRA is proposed to address the molecular initiating event of the skin sensitisation AOP, namely protein reactivity, by quantifying the reactivity of test chemicals towards model synthetic peptides containing either lysine or cysteine (1). Cysteine and lysine percent peptide depletion values are then used to categorise a substance in one of four classes of reactivity for supporting the discrimination between skin sensitisers and non-sensitisers (2).

11. The DPRA test method proved to be transferable to laboratories experienced in high-performance liquid chromatography (HPLC) analysis. The level of reproducibility in predictions that can be expected from the test method is in the order of 85% within laboratories and 80% between laboratories (3). Results generated in the validation study (4) and published studies (5) overall indicate that the accuracy of the DPRA in discriminating sensitisers (i.e. UN GHS Cat. 1) from non-sensitisers is 80% (N=157) with a sensitivity of 80% (88/109) and specificity of 77% (37/48) when compared to LLNA results. The DPRA is more likely to under predict chemicals showing a low to moderate skin sensitisation potency (i.e. UN GHS subcategory 1B) than chemicals showing a high skin sensitisation potency (i.e. UN GHS subcategory 1A) (4) (5). However, the accuracy values given here for the DPRA as a stand-alone test method are only indicative since the test method should be considered in combination with other sources of information in the context of an IATA or a DA and in accordance with the provisions of paragraphs 7 and 8 in the General introduction. Furthermore when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA test as well as other animal tests may not fully reflect the situation in the species of interest, i.e. humans. On the basis of the overall data available, the DPRA was shown to be applicable to test chemicals covering a variety of organic functional groups, reaction mechanisms, skin sensitisation potency (as determined in *in vivo* studies) and physico-chemical properties (1) (2) (3) (5). Taken together, this information indicates the usefulness of the DPRA to contribute to the identification of skin sensitisation hazard.

12. The term "test chemical" is used in this Test Guideline to refer to what is being tested[1] and is not related to the applicability of the DPRA to the testing of substances and/or mixtures. This test method is not applicable for the testing of metal compounds since they are known to react with proteins with mechanisms other than covalent binding. A test chemical should be soluble in an appropriate solvent at a final concentration of 100 mM (see paragraph 10). However, test chemicals that are not soluble at this concentration may still be tested at lower soluble concentrations. In such a case, a positive result could still be used to support the identification of the test chemical as a skin sensitiser but no firm

[1] In June 2013, the Joint Meeting agreed that where possible, a more consistent use of the term “test chemical” describing what is being tested should now be applied in new and updated Test Guidelines.

conclusion on the lack of reactivity should be drawn from a negative result. Limited information is currently available on the applicability of the DPRA to mixtures of known composition (4) (5). The DPRA is nevertheless considered to be technically applicable to the testing of multi-constituent substances and mixtures of known composition (see paragraph 4 and 10). When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Appendix of the Test Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. The current prediction model cannot be used for complex mixtures of unknown composition or for substances of unknown or variable composition, complex reaction products or biological materials (i.e. UVCB substances) due to the defined molar ratio of test chemical and peptide. For this purpose a new prediction model based on a gravimetric approach will need to be developed. In cases where evidence can be demonstrated on the non-applicability of the test method to other specific categories of chemicals, the test method should not be used for those specific categories of chemicals.

13. The test method described in this Appendix of the Test Guideline is an *in chemico* method that does not encompass a metabolic system. Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential (i.e. pro-haptens) cannot be detected by the test method. Chemicals that become sensitisers after abiotic transformation (i.e. pre-haptens) are reported to be in most cases correctly detected by the test method (4) (9) (10). In the light of the above, negative results obtained with the test method should be interpreted in the context of the stated limitations and in the connection with other information sources within the framework of an IATA or a DA. Test chemicals that do not covalently bind to the peptide but promote its oxidation (i.e. cysteine dimerisation) could lead to a potential over estimation of peptide depletion, resulting in possible false positive predictions and/or assignment to a higher reactivity class (see paragraphs 21 and 22).

14. As described, the DPRA assay supports the discrimination between skin sensitisers and non-sensitisers. However, it may also potentially contribute to the assessment of sensitising potency (6) (11) when used in integrated approaches such as IATA or DA (12). However further work, preferably based on human data, is required to determine how DPRA results may possibly inform potency assessment.

PRINCIPLE OF THE TEST

15. The DPRA is an *in chemico* method which quantifies the remaining concentration of cysteine- or lysine-containing peptide following 24 hours incubation with the test chemical at 22.5-30°C. The synthetic peptides contain phenylalanine to aid in the detection. Relative peptide concentration is measured by high-performance liquid chromatography (HPLC) with gradient elution and UV detection at 220 nm. Cysteine- and lysine peptide percent depletion values are then calculated and used in a prediction model (see paragraph 21) which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitisers and non-sensitisers.

16. Prior to routine use of the method described in this Appenix, laboratories should demonstrate technical proficiency, using the ten proficiency substances listed in Annex 1.

PROCEDURE

17. This test method is based on the DPRA DB-ALM protocol nº 154 (7) which represents the protocol used for the EURL ECVAM-coordinated validation study. It is recommended that this protocol is used when implementing and using the method in the laboratory. The following is a description of the main components and procedures for the DPRA. If an alternative HPLC set-up is used, its equivalence to the validated set-up described in the DB-ALM protocol should be demonstrated (e.g. by testing the proficiency substances in Annex 1).

Preparation of the cysteine or lysine-containing peptides

18. Stock solutions of cysteine (Ac-RFAA**C**AA-COOH) and lysine (Ac-RFAA**K**AA-COOH) containing synthetic peptides of purity higher than 85% and preferably > 90%, should be freshly prepared just before their incubation with the test chemical. The final concentration of the cysteine peptide should be 0.667 mM in pH 7.5 phosphate buffer whereas the final concentration of the lysine peptide should be 0.667 mM in pH 10.2 ammonium acetate buffer. The HPLC run sequence should be set up in order to keep the HPLC analysis time less than 30 hours. For the HPLC set up used in the validation study and described in this test method, up to 26 analysis samples (which include the test chemical, the positive control and the appropriate number of solvent controls based on the number of individual solvents used in the test, each tested in triplicate), can be accommodated in a single HPLC run. All of the replicates analysed in the same run should use the identical cysteine and lysine peptide stock solutions. It is recommended to prove individual peptide batches for proper solubility prior to their use.

Preparation of the test chemical

19. Solubility of the test chemical in an appropriate solvent should be assessed before performing the assay following the solubilisation procedure described in the DPRA DB-ALM protocol (7). An appropriate solvent will dissolve the test chemical completely. Since in the DPRA the test chemical is incubated in large excess with either the cysteine or the lysine peptides, visual inspection of the forming of a clear solution is considered sufficient to ascertain that the test chemical (and all of its components in the case of testing a multi-constituent substance or a mixture) is dissolved. Suitable solvents are, acetonitrile, water, 1:1 mixture water:acetonitrile, isopropanol, acetone or 1:1 mixture acetone:acetonitrile. Other solvents can be used as long as they do not have an impact on the stability of the peptide as monitored with reference controls C (i.e. samples constituted by the peptide alone dissolved in the appropriate solvent; see Annex 2). If the test chemical is not soluble in any of the solvents mentioned above, DMSO can be used as a last resort and in minimal amounts. It is important to note that DMSO may lead to peptide dimerisation and as a result, it may be more difficult to meet the acceptance criteria. If DMSO is chosen, attempts should be made to first solubilise the test chemical in 300 µL of DMSO and dilute the resulting solution with 2700 µL of acetonitrile. If the test chemical is not soluble in this mixture, attempts should be made to solubilise the same amount of test chemicals in 1500 µL of DMSO and dilute the resulting solution with 1500 µL of acetonitrile. The test chemical should be pre-weighed into glass vials and dissolved immediately before testing in an appropriate solvent to prepare a 100 mM solution. For mixtures and multi-constituent substances of known composition, a single purity should be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight should be determined by considering the individual molecular weights of each component

in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight should then be used to calculate the weight of test chemical necessary to prepare a 100 mM solution. For polymers for which a predominant molecular weight cannot be determined, the molecular weight of the monomer (or the apparent molecular weight of the various monomers constituting the polymer) may be considered to prepare a 100 mM solution. However, when testing mixtures, multi-constituent substances or polymers of known composition, it should be considered to also test the neat chemical. For liquids, the neat chemical should be tested as such without any prior dilution by incubating it at 1:10 and 1:50 ratio with the cysteine and lysine peptides, respectively. For solids, the test chemical should be dissolved to its maximum soluble concentration in the same solvent used to prepare the apparent 100 mM solution. It should then be tested as such without any further dilution by incubating it at 1:10 and 1:50 ratio with the cysteine and lysine peptides, respectively. Concordant results (reactive or non-reactive) between the apparent 100 mM solution and the neat chemical should allow for a firm conclusion on the result.

Preparation of the positive control, reference controls and coelution controls

20. Cinnamic aldehyde (CAS 104-55-2; ≥95% food-grade purity) should be used as positive control (PC) at a concentration of 100 mM in acetonitrile. Other suitable positive controls providing mid-range depletion values may be used if historical data are available to derive comparable run acceptance criteria. In addition reference controls (i.e. samples containing only the peptide dissolved in the appropriate solvent) should also be included in the HPLC run sequence and these are used to verify the HPLC system suitability prior to the analysis (reference controls A), the stability of the reference controls over time (reference control B) and to verify that the solvent used to dissolve the test chemical does not impact the percent peptide depletion (reference control C) (see Annex 2). The appropriate reference control for each substance is used to calculate the percent peptide depletion for that substance (see paragraph 18). In addition, a co-elution control constituted by the test chemical alone for each of the test chemicals analysed should be included in the run sequence to detect possible co-elution of the test chemical with either the lysine or the cysteine peptide.

Incubation of the test chemical with the cysteine and lysine peptide solutions

21. Cysteine and lysine peptide solutions should be incubated in glass autosampler vials with the test chemical at 1:10 and 1:50 ratio respectively. If a precipitate is observed immediately upon addition of the test chemical solution to the peptide solution, due to low aqueous solubility of the test chemical, one cannot be sure how much test chemical remained in the solution to react with the peptide. Therefore, in such a case, a positive result could still be used, but a negative result is uncertain and should be interpreted with due care (see also provisions in paragraph 10 for the testing of chemicals not soluble up to a concentration of 100 mM). The reaction solution should be left in the dark at 22.5-30°C for 24±2 hours before running the HPLC analysis. Each test chemical should be analysed in triplicate for both peptides. Samples have to be visually inspected prior to HPLC analysis. If a precipitate or phase separation is observed, samples may be centrifuged at low speed (100-400xg) to force precipitate to the bottom of the vial as a precaution since large amounts of precipitate may clog the HPLC tubing or columns. If a precipitation or phase separation is observed after the incubation period, peptide depletion may be underestimated and a conclusion on the lack of reactivity cannot be drawn with sufficient confidence in case of a negative result.

Preparation of the HPLC standard calibration curve

22. A standard calibration curve should be generated for both the cysteine and the lysine peptides. Peptide standards should be prepared in a solution of 20% or 25% acetonitrile:buffer using phosphate buffer (pH 7.5) for the cysteine peptide and ammonium acetate buffer (pH 10.2) for the lysine peptide. Using serial dilution standards of the peptide stock solution (0.667 mM), 6 calibration solutions should be prepared to cover the range from 0.534 to 0.0167 mM. A blank of the dilution buffer should also be included in the standard calibration curve. Suitable calibration curves should have an $r^2>0.99$.

HPLC preparation and analysis

23. The suitability of the HPLC system should be verified before conducting the analysis. Peptide depletion is monitored by HPLC coupled with an UV detector (photodiode array detector or fixed wavelength absorbance detector with 220 nm signal). The appropriate column is installed in the HPLC system. The HPLC set-up described in the validated protocol uses a Zorbax SB-C-18 2.1 mm x 100 mm x 3.5 micron as preferred column. With this reversed-phase HPLC column, the entire system should be equilibrated at 30°C with 50% phase A (0.1% (v/v) trifluoroacetic acid in water) and 50% phase B (0.085% (v/v) trifluoroacetic acid in acetonitrile) for at least 2 hours before running. The HPLC analysis should be performed using a flow rate of 0.35 mL/min and a linear gradient from 10% to 25% acetonitrile over 10 minutes, followed by a rapid increase to 90% acetonitrile to remove other materials. Equal volumes of each standard, sample and control should be injected. The column should be re-equilibrated under initial conditions for 7 minutes between injections. If a different reversed-phase HPLC column is used, the set-up parameters described above may need to be adjusted to guarantee an appropriate elution and integration of the cysteine and lysine peptides, including the injection volume, which may vary according to the system used (typically in the range from 3-10 µL). Importantly, if an alternative HPLC set-up is used, its equivalence to the validated set-up described above should be demonstrated (e.g. by testing the proficiency substances in Annex 1). Absorbance is monitored at 220 nm. If a photodiode array detector is used, absorbance at 258 nm should also be recorded. It should be noted that some supplies of acetonitrile could have a negative impact on peptide stability and this has to be assessed when a new batch of acetonitrile is used. The ratio of the 220 peak area and the 258 peak area can be used as an indicator of co-elution. For each sample a ratio in the range of 90%<mean[2] area ratio of control samples<100% would give a good indication that co-elution has not occurred.

24. There may be test chemicals which could promote the oxidation of the cysteine peptide. The peak of the dimerised cysteine peptide may be visually monitored. If dimerisation appears to have occurred, this should be noted as percent peptide depletion may be over-estimated leading to false positive predictions and/or assignment to a higher reactivity class (see paragraphs 21 and 22).

25. The HPLC analysis should be timed to assure that the injection of the first sample starts 22 to 26 hours after the test chemical was mixed with the peptide solution. The HPLC run sequence should be set up in order to keep the HPLC analysis time less than 30 hours. For the HPLC set up used in the validation study and described in this test method, up to 26 analysis samples can be accommodated in a single HPLC run (see also paragraph 9). An example of HPLC analysis sequence is provided in Annex 2.

[2] For mean it is meant arithmetic mean throughout the document.

DATA AND REPORTING

Data evaluation

26. The concentration of cysteine or lysine peptide is photometrically determined at 220 nm in each sample by measuring the peak area (area under the curve, AUC) of the appropriate peaks and by calculating the concentration of peptide using the linear calibration curve derived from the standards.

27. The percent peptide depletion is determined in each sample by measuring the peak area and dividing it by the mean peak area of the relevant reference controls C (see Annex 2) according to the formula described below.

$$\text{Percent peptide depletion} = \left[1 - \left(\frac{\text{Peptide peak area in replicate injection}}{\text{Mean peptide peak area in reference controls } C}\right)\right] \times 100$$

Acceptance criteria

28. The following criteria should be met for a run to be considered valid:

a) the standard calibration curve should have an r^2>0.99,

b) the mean percent peptide depletion value of the three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for the cysteine peptide and between 40.2% and 69.0% for the lysine peptide (for other positive controls a reference range needs to be established) and the maximum standard deviation (SD) for the positive control replicates should be <14.9% for the percent cysteine depletion and <11.6% for the percent lysine depletion and

c) the mean peptide concentration of reference controls A should be 0.50±0.05 mM and the coefficient of variation (CV) of peptide peak areas for the nine reference controls B and C in acetonitrile should be <15.0%.

If one or more of these criteria is not met the run should be repeated.

29. The following criteria should be met for a test chemical's results to be considered valid:

a) the maximum standard deviation for the test chemical replicates should be <14.9% for the percent cysteine depletion and <11.6% for the percent lysine depletion,

b) the mean peptide concentration of the three reference controls C in the appropriate solvent should be 0.50±0.05 mM.

If these criteria are not met the data should be rejected and the run should be repeated for that specific test chemical.

Prediction model

30. The mean percent cysteine and percent lysine depletion value is calculated for each test chemical. Negative depletion is considered as "0" when calculating the mean. By using the cysteine 1:10/lysine 1:50 prediction model shown in Table 1, the threshold of 6.38%

average peptide depletion should be used to support the discrimination between skin sensitisers and non-sensitisers in the framework of an IATA or DA. Application of the prediction model for assigning a test chemical to a reactivity class (i.e. low, moderate and high reactivity) may perhaps prove useful to inform potency assessment within the framework of an IATA or DA.

Table 1: Cysteine 1:10/lysine 1:50 prediction model[1]

Mean of cysteine and lysine % depletion	Reactivity Class	DPRA Prediction[2]
0% ≤ mean % depletion ≤ 6.38%	No or minimal reactivity	Negative
6.38% < mean % depletion ≤ 22.62%	Low reactivity	Positive
22.62% < mean % depletion ≤ 42.47%	Moderate reactivity	
42.47% < mean % depletion ≤ 100%	High reactivity	

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.
[2] A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 4.

31. There might be cases where the test chemical (the substance or one or several of the components of a multi-constituent substance or a mixture) absorbs significantly at 220 nm and has the same retention time of the peptide (co-elution). Co-elution may be resolved by slightly adjusting the HPLC set-up in order to further separate the elution time of the test chemical and the peptide. If an alternative HPLC set-up is used to try to resolve co-elution, its equivalence to the validated set-up should be demonstrated (e.g. by testing the proficiency substances in Annex 1). When co-elution occurs the peak of the peptide cannot be integrated and the calculation of the percent peptide depletion is not possible. If co-elution of such test chemicals occurs with both the cysteine and the lysine peptides then the analysis should be reported as "inconclusive". In cases where co-elution occurs only with the lysine peptide, then the cysteine 1:10 prediction model reported in Table 2 can be used.

Table 2: Cysteine 1:10 prediction model[1]

Cysteine (Cys) % depletion	Reactivity class	DPRA prediction[2]
0% ≤ Cys % depletion ≤ 13.89%	No or minimal reactivity	Negative
13.89% < Cys % depletion ≤ 23.09%	Low reactivity	Positive
23.09% < Cys % depletion ≤ 98.24%	Moderate reactivity	
98.24% < Cys % depletion ≤ 100%	High reactivity	

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.
[2] A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 4.

32. There might be other cases where the overlap in retention time between the test chemical and either of the peptides is incomplete. In such cases percent peptide depletion values can be estimated and used in the cysteine 1:10/lysine 1:50 prediction model, however assignment of the test chemical to a reactivity class cannot be made with accuracy.

33. A single HPLC analysis for both the cysteine and the lysine peptide should be sufficient for a test chemical when the result is unequivocal. However, in cases of results close to the threshold used to discriminate between positive and negative results (i.e. borderline results), additional testing may be necessary. If situations where the mean percent depletion falls in the range of 3% to 10% for the cysteine 1:10/lysine 1:50 prediction model or the cysteine percent depletion falls in the range of 9% to 17% for the cysteine 1:10 prediction model, a second run may be considered, as well as a third one in case of discordant results between the first two runs.

Test report

34. The test report should include the following information

Test chemical

- Mono-constituent substance
    - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
    - Physical appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
    - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
    - Treatment prior to testing, if applicable (e.g. warming, grinding);
    - Concentration(s) tested;
    - Storage conditions and stability to the extent available.
- Multi-constituent substance, UVCB and mixture:
    - Characterisation as far as possible by e.g. chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;
    - Physical appearance, water solubility and additional relevant physicochemical properties, to the extent available;
    - Molecular weight or apparent molecular weight in case of mixtures/polymers of known compositions or other information relevant for the conduct of the study;
    - Treatment prior to testing, if applicable (e.g. warming, grinding);
    - Concentration(s) tested;
    - Storage conditions and stability to the extent available.

Controls

- Positive control
    - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;

    - Physical appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
    - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
    - Treatment prior to testing, if applicable (e.g. warming, grinding);
    - Concentration(s) tested;
    - Storage conditions and stability to the extent available;
    - Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.
- Solvent/vehicle
    - Solvent/vehicle used and ratio of its constituents, if applicable;
    - Chemical identification(s), such as IUPAC or CAS name(s), CAS number(s), and/or other identifiers;
    - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
    - Physical appearance, molecular weight, and additional relevant physicochemical properties in the case other solvents/vehicles than those mentioned in the test method are used and to the extent available;
    - Storage conditions and stability to the extent available;
    - Justification for choice of solvent for each test chemical;
    - For acetonitrile, results of test of impact on peptide stability.

Preparation of peptides, positive control and test chemical

- Characterisation of peptide solutions (supplier, lot, exact weight of peptide, volume added for the stock solution);
- Characterisation of positive control solution (exact weight of positive control substance, volume added for the test solution);
- Characterisation of test chemical solutions (exact weight of test chemical, volume added for the test solution).

HPLC instrument setting and analysis

- Type of HPLC instrument, HPLC and guard columns, detector, autosampler;
- Parameters relevant for the HPLC analysis such as column temperature, injection volumes, flow rate and gradient.

System suitability

- Peptide peak area at 220 nm of each standard and reference control A replicate;
- Linear calibration curve graphically represented and the r^2 reported;
- Peptide concentration of each reference control A replicate;

- Mean peptide concentration (mM) of the three reference controls A, SD and CV;
- Peptide concentration of reference controls A and C.

Analysis sequence

- For reference controls:
    - Peptide peak area at 220 nm of each B and C replicate;
    - Mean peptide peak area at 220 nm of the nine reference controls B and C in acetonitrile, SD an CV (for stability of reference controls over analysis time);
    - For each solvent used, the mean peptide peak area at 220 nm of the three appropriate reference controls C (for the calculation of percent peptide depletion);
    - For each solvent used, the peptide concentration (mM) of the three appropriate reference controls C;
    - For each solvent used, the mean peptide concentration (mM) of the three appropriate reference controls C, SD and CV.
- For positive control:
    - Peptide peak area at 220 nm of each replicate;
    - Percent peptide depletion of each replicate;
    - Mean percent peptide depletion of the three replicates, SD and CV.
- For each test chemical:
    - Appearance of precipitate in the reaction mixture at the end of the incubation time, if observed. If precipitate was re-solubilised or centrifuged;
    - Presence of co-elution;
    - Description of any other relevant observations, if applicable;
    - Peptide peak area at 220 nm of each replicate;
    - Percent peptide depletion of each replicate;
    - Mean of percent peptide depletion of the three replicate, SD and CV;
    - Mean of percent cysteine and percent lysine depletion values;
    - Prediction model used and DPRA prediction.

Proficiency testing

- If applicable, the procedure used to demonstrate proficiency of the laboratory in performing the test method (e.g. by testing of proficiency substances) or to demonstrate reproducible performance of the test method over time.

Discussion of the results

- Discussion of the results obtained with the DPRA test method;

- Discussion of the test method results in the context of an IATA if other relevant information is available.

Conclusion

LITERATURE FOR APPENDIX I

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APPENDIX I, ANNEX 1

PROFICIENCY SUBSTANCES

In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay

Prior to routine use of the test method described in this test method, laboratories should demonstrate technical proficiency by correctly obtaining the expected DPRA prediction for the 10 proficiency substances recommended in Table 1 and by obtaining cysteine and lysine depletion values that fall within the respective reference range for 8 out of the 10 proficiency substances for each peptide. These proficiency substances were selected to represent the range of responses for skin sensitisation hazards. Other selection criteria were that they are commercially available, that high quality *in vivo* reference data and high quality *in vitro* data generated with the DPRA are available, and that they were used in the EURL ECVAM-coordinated validation study to demonstrate successful implementation of the test method in the laboratories participating in the study.

Table 1: Recommended proficiency substances for demonstrating technical proficiency with the Direct Peptide Reactivity Assay

Proficiency substances	CASRN	Physical state	*In vivo* prediction[1]	DPRA prediction[2]	Range[3] of % cysteine peptide depletion	Range[3] of % lysine peptide depletion
2,4-Dinitrochlorobenzene	97-00-7	Solid	Sensitiser (extreme)	Positive	90-100	15-45
Oxazolone	15646-46-5	Solid	Sensitiser (extreme)	Positive	60-80	10-55
Formaldehyde	50-00-0	Liquid	Sensitiser (strong)	Positive	30-60	≤24
Benzylideneacetone	122-57-6	Solid	Sensitiser (moderate)	Positive	80-100	≤7
Farnesal	19317-11-4	Liquid	Sensitiser (weak)	Positive	15-55	≤25
2,3-Butanedione	431-03-8	Liquid	Sensitiser (weak)	Positive	60-100	10-45
1-Butanol	71-36-3	Liquid	Non-sensitiser	Negative	≤7	≤5.5
6-Methylcoumarin	92-48-8	Solid	Non-sensitiser	Negative	≤7	≤5.5
Lactic Acid	50-21-5	Liquid	Non-sensitiser	Negative	≤7	≤5.5
4-Methoxyacetophenone	100-06-1	Solid	Non-sensitiser	Negative	≤7	≤5.5

[1]The *in vivo* hazard and (potency) predictions are based on LLNA data (5). The *in vivo* potency is derived using the criteria proposed by ECETOC (8).

[2] A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 4.

[3] Ranges determined on the basis of at least 10 depletion values generated by 6 independent laboratories.

APPENDIX I, ANNEX 2

EXAMPLES OF ANALYSIS SEQUENCE

Calibration standards and reference controls	STD1 STD2 STD3 STD4 STD5 STD6 Dilution buffer Reference control A, rep 1 Reference control A, rep 2 Reference control A, rep 3
Co-elution controls	Co-elution control 1 for test chemical 1 Co-elution control 2 for test chemical 2
Reference controls	Reference control B, rep 1 Reference control B, rep 2 Reference control B, rep 3
First set of replicates	Reference control C, rep 1 Cinnamic aldehyde, rep 1 Sample 1, rep 1 Sample 2, rep 1
Second set of replicates	Reference control C, rep 2 Cinnamic aldehyde, rep 2 Sample 1, rep 2 Sample 2, rep 2
Third set of replicates	Reference control C, rep 3 Cinnamic aldehyde, rep 3 Sample 1, rep 3 Sample 2, rep 3
Reference controls	Reference control B, rep 4 Reference control B, rep 5 Reference control B, rep 6

Three sets of reference controls (i.e. samples constituted only by the peptide dissolved in the appropriate solvent) should be included in the analysis sequence:
Reference control A: used to verify the suitability of the HPLC system.
Reference control B: included at the beginning and at the end of the analysis sequence to verify stability of reference controls over the analysis time.
Reference control C: included in the analysis sequence to verify that the solvent used to dissolve the test chemical does not impact the percent peptide depletion.

APPENDIX II

In Chemico Skin Sensitisation: Amino acid Derivative Reactivity Assay (ADRA)

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

35. The ADRA is proposed to address the molecular initiating event of the skin sensitisation AOP—namely, protein reactivity—by quantifying the reactivity of test chemicals towards model synthetic amino acid derivatives containing either lysine or cysteine (1) (2) (3). Depletion values of cysteine and lysine derivatives are then used to support the discrimination between skin sensitisers and non-sensitisers (1) (2) (3).

36. The ADRA proved to be transferable to laboratories experienced in high-performance liquid chromatography (HPLC) analysis. ADRA's WLR was 100% (10/10), 100% (7/7), 90% (9/10), and 100% (10/10) in four participating laboratories. BLR for 40 test chemicals calculated based the results from three participating laboratories was 91.9% (4). For the 40 chemicals tested in the validation study in four laboratories, the cumulative accuracy was 86.9% (139/160), sensitivity was 81.5% (88/108), and specificity was 98.1% (51/52) (4) (5). Results from the validation study (4) (5) as well as from other published studies (3) indicate that ADRA identified sensitisers and non-sensitisers with an accuracy of 79% (98/124) (124 compounds that fall within ADRA's applicability domain), a sensitivity of 74% (65/88), and a specificity of 92% (33/36) relative to LLNA results (6). In addition, the prediction of human skin sensitisation for 73 compounds that fall within ADRA's applicability domain has an accuracy of 86% (63/73), a sensitivity of 85% (44/52), and a specificity of 90% (19/21) (6). However, the accuracy values given here for ADRA as a stand-alone test method are for reference only, since it is recommended that the test method be used in combination with other sources of information in the context of an IATA and in accordance with the provisions of paragraphs 7 and 8 in the General Introduction. Furthermore when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA test as well as other animal tests may not fully reflect the situation in the species of interest, which is humans. On the basis of the overall data available, ADRA's applicability domain was shown to include a variety of organic functional groups, reaction mechanisms, skin sensitisation potencies (as determined in *in vivo* studies), and physicochemical properties (1) (2) (3) (4). Following an independent peer review, the ADRA validation study was considered to demonstrate that this method should be acceptable as part of an integrated testing strategy for the predictive identification of skin sensitisation hazard (7).

37. The term "test chemical" is used in this Test Guideline to refer to what is being tested and is not related to the applicability of the ADRA to the testing of substances and/or mixtures. This test method is not applicable to the testing of metal compounds, which are known to react with proteins via mechanisms other than covalent binding. The test method described in this Appendix of the Test Guideline is an *in chemico* method that does not encompass a metabolic system. Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential (i.e. pro-haptens) cannot be detected by the test method. Chemicals that become sensitisers after abiotic transformation (i.e. pre-haptens) are

reported to be in some cases correctly detected by the test method (1) (2) (3) (4). In the light of the above, negative results obtained with the test method should be interpreted in the context of the stated limitations and in the connection with other information sources within the framework of an IATA. Test chemicals that promote the oxidation of the *N*-(2-(1-naphthyl)acetyl)-L-cysteine (NAC) reagent (i.e. cysteine dimerisation) could lead to a potential over-estimation of NAC depletion, resulting in possible false positive predictions (see paragraphs 27 and 28); it may be possible to detect and quantify any NAC dimer formed by HPLC, thus confirming or ruling out that the NAC reagent has been depleted via oxidative dimerisation as opposed to reaction and covalent bonding to the test item substance(s).

38. The ADRA test method allows testing of poorly soluble chemicals. To be tested, a test chemical should be soluble in an appropriate solvent at a final concentration of 1 mM (see paragraph 15). Test chemicals that are not soluble at this concentration may still be tested at lower concentrations. In such cases, a positive result could still be used to support identification of the test chemical as a skin sensitiser but no firm conclusion on the lack of reactivity should be drawn from a negative result.

39. In general, many organic compounds absorb UV in the range of 220 nm. In the case of co-elution of the nucleophilic reagent and the test chemical, this might result in false negative prediction. This may happen with the DPRA which specifies that quantification of the peptide-based nucleophilic reagents has to be performed at 220 nm. In contrast to this, the nucleophilic reagents used in ADRA are quantified at 281 nm. The substances that absorb UV in this range of the spectrum are generally limited to those having conjugated double bonds, which significantly lowers the potential for co-elution (8).

40. The current prediction model cannot be used for complex mixtures of unknown composition or for substances of unknown or variable composition, complex reaction products, or biological materials (UVCB substances) due to the need for defined molar ratio of test chemical and nucleophilic reagents. Limited information is currently available on the applicability of the ADRA to mixtures (9) (10). A new protocol has to be developed for multi-constituent substances and mixtures to be used with test methods like ADRA, which utilise HPLC analysis to quantify the depletion of nucleophilic reagents (9) (10). Thus, although it is impossible to define fixed methods in this guideline, which can evaluate multi-constituent substances and mixtures, paragraph 16 describes an evaluation method that is considered to be applicable at the present time for multi-constituent substances or mixtures of known composition (9). Such substances were however not tested during the validation studies. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically.

41. ADRA can be used to support the discrimination between skin sensitisers and non-sensitisers. Further work, preferably based on human data, is necessary to determine whether ADRA results can contribute to potency assessment when considered in combination with other information sources.

PRINCIPLE OF THE TEST

42. ADRA is an *in chemico* test method that quantifies residual concentrations of the cysteine derivative *N*-(2-(1-naphthyl)acetyl)-*L*-cysteine (CAS. 32668-00-1), which is known as NAC, and the lysine derivative α-*N*-(2-(1-naphthyl)acetyl)-*L*-lysine (CAS. 397841-92-8), known as NAL, following a 24±1 hour incubation at 25±1ºC in the presence of a test chemical. Both these derivatives include a naphthalene ring that is introduced to their *N*-terminal in order to facilitate UV detection. The relative concentrations of NAC and NAL are measured by high-performance liquid chromatography (HPLC) with gradient elution and UV detection at 281 nm. Percent depletion values are then calculated for both NAC and NAL and compared to a prediction model (see paragraph 26).

43. Prior to routine use of the method described in this test method, laboratories should demonstrate technical proficiency, using the ten proficiency substances listed in Annex 1 of this Appendix.

PROCEDURE

44. This test method is based on the protocol (11) used for the JaCVAM-coordinated ADRA validation study and is recommended for use when implementing ADRA at a laboratory. The main components and procedures for the ADRA are described below. Before using an alternative HPLC set-up, its equivalence to the validated set-up described in the protocol should be demonstrated, preferably by testing the proficiency substances in Annex 1 of this Appendix.

Quality of NAC and NAL

45. The Nucleophilic Reagents can be obtained as an ADRA Kit for Skin Sensitisation Test, from FUJIFILM Wako (FFWK) Pure Chemical Corporation, Catalog No. 296-80901. Manufacturing NAC/NAL is patented in Japan only, by Fujifilm Corporation. Therefore, manufacturers in other countries can produce NAC/NAL without permission. In case other NAC/NAL are used, these should satisfy three quality criteria described below. Quality checks can be obviated and ADRA testing performed without delay by purchasing NAC and NAL that have been manufactured specifically to satisfy these quality criteria.

Quality required for NAC and NAL:

1) Purity: Both NAC and NAL are to be at least 98% pure.

2) Stability: Using NAC and NAL stock solution, prepare a reference control free of any test chemical and quantify the residual levels of NAC and NAL both immediately after preparation (0 hours) and after a 24 hour incubation. Residual levels of NAC and NAL are to be a minimum of 90% in either case (11). The residual level of NAC is calculated as a percentage of the sum of NAC and the residual level of NAC dimers.

3) Reactivity: NAC and NAL are to be evaluated for reactivity with the ten proficiency substances given in Annex 1 and should satisfy the requirement given therein.

Preparation of the NAC and NAL stock solution

46. The solubility of individual NAC and NAL batches should be verified prior to use. NAC stock solution should be prepared to a concentration of 2 mM in 100 mM of pH 8.0 phosphate buffer, including 0.333 μM of EDTA, as well as NAL stock solution to a concentration of 2 mM in 100 mM of pH 10.2 phosphate buffer. These two stock solutions are then diluted in buffer to prepare 6.667 μM stock solutions. Both NAC and NAL stock solutions should be used as soon as possible after preparation (3). In the event that they are to be stored, these stock solutions may be frozen and stored for up to twelve months time at less than -75°C prior to use. The final concentration of the NAC solution is 5 μM in pH 8.0 phosphate buffer, and the final concentration of the NAL solution is 5 μM in pH 10.2 phosphate buffer.

Preparation of the test chemical solution

47. Solubility of the test chemical in an appropriate solvent should be assessed before performing the assay in accordance with the solubilisation procedure described in the ADRA JaCVAM protocol (11). An appropriate solvent should dissolve the test chemical completely. Since the ADRA protocol stipulates that the test chemical be incubated in an excess volume of both NAC and NAL, visual inspection of the clear test chemical solution is considered sufficient to confirm that the test chemical (and all its constituents, if testing a multi-constituent substance or a mixture) is dissolved. Suitable solvents are distilled water, acetonitrile and acetone. If the test chemical is not soluble in any of the solvents mentioned above, DMSO can be used as a last resort and in minimal amounts. It is important to note that DMSO may lead to dimerisation of the nucleophilic reagent NAC (12) and as a result, it may be more difficult to meet the acceptance criteria. If DMSO is chosen, attempts should be made to solubilise the test chemical in a 1:20 mixture of DMSO and acetonitrile (5% DMSO in acetonitrile). When using a DMSO-acetonitrile solvent, the test chemical should be dissolved in DMSO, and then this solution should be diluted 20-fold with acetonitrile to prepare a 1 mM test chemical solution. In case the use of DMSO leads to increased dimerisation of the NAC reagent, this can be checked analytically as the NAC dimer can be detected by HPLC. The test chemical should be pre-weighed into a disposable polypropylene tube and dissolved immediately before testing in an appropriate solvent to prepare a 1 mM solution.

48. Mono-constituent substances of unknown molecular weight may be tested in a test chemical solution at a concentration of 0.5 mg/mL rather than 1 mM (9). Polymers which are well characterised should also be tested at a concentration of 1 mM based on the mean number average molecular weight, in a manner analogous to the procedure for mono-constituent compounds.

49. Mixtures and multi constituent substances, of known composition are to be tested as follows:

> 1) Liquids: Generally, tested as an undiluted mixture. In cases where low solubility of the test item prevents formation of reaction solution, i.e. undissolved material, clouding, and/or precipitation is observed, a positive result may still be used in the assessment, whereas a negative result is uncertain and should be interpreted with due care. Insofar as results could be false positives, however, predictions should be interpreted with due care.
>
> 2) Solids: The test chemical should be dissolved to maximum soluble concentration in the same solvent used to prepare the 1 mM test chemical solution. The test

chemical solution of the highest concentration possible is then tested as an undiluted mixture. In cases where low solubility of the test item prevents formation of reaction solution, i.e. undissolved material, clouding, and/or precipitation is observed, a positive result may still be used in the assessment, whereas a negative result is uncertain and should be interpreted with due care. Insofar as results could be false positives, however, predictions should be interpreted with due care.

Preparation of the positive control, reference controls and co-elution controls

50. Phenylacetaldehyde (CAS 122-78-1, ≥90% purity) should be used as positive control (PC) at a concentration of 1 mM in acetonitrile. Other suitable positive controls that provide mid-range depletion values may be used if historical data are available to derive comparable run acceptance criteria. In addition, reference controls comprising only NAC or only NAL dissolved in the appropriate solvent should also be included in the HPLC run sequence, so they can be used to verify the HPLC system suitability prior to analysis (Reference Control A), the stability of the reference controls over time (Reference Control B), and any effects of the solvent used on depletion of NAC or NAL (Reference Control C) (See Annex 2). The percent NAC and NAL depletion for a test chemical is calculated using an appropriate reference control for that test chemical (see paragraph 23). Also, a co-elution control comprising only the test chemical should be included in the run sequence to detect possible co-elution of the test chemical with either the NAC or NAL.

Incubation of the test chemical with the NAC and NAL solutions

51. Both the NAC and the NAL solutions should be incubated with the test chemical at 1:50 ratio in a 96-well microplate. The observation of precipitate immediately upon addition of the test chemical solution to the NAC and the NAL solutions is an indication of poor solubility, which means that there is no way to know exactly how much test chemical is contained in the solution. Thus, although positive results can be used with confidence, negative results are uncertain and should be interpreted with due care (see also paragraph 4 regarding the testing of chemicals not soluble at concentrations as high as 1 mM). The reaction solution should be incubated in the dark at 25±1ºC for 24±1 hours before performing HPLC analysis. After incubation, trifluoroacetic acid (TFA) (≥ 98%) should be added as a fixing solution to stop the reaction (3).

HPLC preparation and analysis

52. Each test chemical should be analysed in triplicate to determine percent depletion for both NAC and NAL. Although adding the fixing solution does stop the reaction, measurement of the reaction solution is to be performed as soon as possible and in any case within three days after adding the fixing solution. For example, when HPLC analysis of NAC and NAL are performed separately using two 96-well microplates, up to 34 samples may be analysed at one time, including the test chemical, the positive control, and the appropriate number of solvent controls based on the number of individual solvents used in the test, each in triplicate. All of the replicates analysed in a single run should use identical batches of NAC and NAL stock solution. Test chemical and control solutions are to be visually inspected prior to HPLC analysis and may be centrifuged at low speed (100–400 × g) to force any precipitate to the bottom of the vial as a precaution against large amounts of precipitate clogging the HPLC tubing or columns. Observation of precipitation or phase separation after the incubation period is an indication that NAC and NAL depletion could be misleading, and negative results in that case are uncertain and should be interpreted with

due care, as well as for any precipitate observed at the beginning of the incubation period (see above).

53. A standard calibration curve should be generated for both NAC and NAL. Standard solutions of both NAC and NAL should be prepared in 20% acetonitrile in buffer and containing 0.5% trifluoroacetic acid. For NAC, a phosphate buffer at pH 8.0, and for NAL, a phosphate buffer at pH 10.2 should be used. Serial dilution of the NAC and NAL stock solutions (5.0 μM) will be used to prepare six calibration solutions in concentrations from 5.0 to 0.156 μM as well as a blank of the dilution buffer. Suitable calibration curves should have an $R^2 > 0.990$.

54. The suitability of the HPLC system should be verified before conducting the analysis. Both NAC and NAL depletion is monitored by HPLC coupled with an UV detector (photodiode array detector or fixed wavelength absorbance detector with 281 nm signal). The appropriate column is installed in the HPLC system. The recommended HPLC set-up described in the validated protocol uses a column (Base particle: core-shell type silica gel, Particle size: 2.5~2.7 μm, colomn size: 3.0 × 150 mm) as preferred column. With this reversed-phase HPLC column, the entire system should be equilibrated for at least 30 minutes at 40ºC with 50% phase A (0.1% (v/v) trifluoroacetic acid in water), 50% phase B (0.1% (v/v) trifluoroacetic acid in acetonitrile) before use. Then, the column is conditioned by running the gradient at least twice before actual use. The HPLC analysis should be performed using a flow rate of 0.30 mL/min and a linear gradient from 30% to 55% acetonitrile for NAC and from 25% to 45% acetonitrile for NAL within 10 minutes, followed by a rapid increase to 100% acetonitrile to remove other materials. Equal volumes of the standard solutions, test chemical solutions, and control solutions should be injected. The column should be re-equilibrated under initial conditions for 6.5 minutes between injections. If a different reversed-phase HPLC column is used, the set-up parameters described above may need to be adjusted to guarantee an appropriate elution and integration of the NAC and NAL, including the injection volume, which may vary according to the system used (typically in the range from 10–20 μL). Importantly, if an alternative HPLC set-up is used, its equivalence to the validated set-up described above should be demonstrated, preferably by testing the proficiency substances in Annex 1. Absorbance is monitored at 281 nm. If a photodiode array detector is used, absorbance at 291 nm should also be recorded. It should be noted that some batches of acetonitrile could have a negative impact on NAC and NAL stability and this has to be assessed when a new batch of acetonitrile is used. The ratio of the 281 nm peak area and the 291 nm peak area can be used as an indicator of co-elution. For each sample a ratio in the range of 90% < mean area ratio of control samples < 100% would give a good indication that co-elution has not occurred. An example of HPLC analysis sequence is provided in Annex 2.

55. There are some test chemicals that could potentially promote oxidation of NAC. The peak of the dimerised NAC may be monitored visually. Any apparent dimerisation should be noted, since overestimation of NAC depletion could result in false-positive predictions (See paragraphs 26 and 27).

DATA AND REPORTING

Data evaluation

56. The concentration of both NAC and NAL is photometrically determined at 281 nm in each sample by measuring the peak area (area under the curve, AUC) of the appropriate peaks and by calculating the concentration of both NAC and NAL using the linear calibration curve derived from the standards.

57. The percent depletion for both NAC and NAL is determined in each sample by measuring the peak area and dividing it by the mean peak area of the relevant Reference Controls C (See Annex 2) according to the formula described below.

$$\text{Percent NAC or NAL depletion} = \left[1-\left[\frac{\text{NAC or NAL peak area in replicate injection}}{\text{Mean NAC or NAL peak area in reference controls C}}\right]\right]\text{x}100$$

Acceptance criteria

58. The following criteria should be met:

a) the standard calibration curve should have an $R^2 > 0.990$,

b) the mean percent NAC and NAL depletion value of the three replicates for the positive control phenylacetaldehyde should be between 6% and 30% for NAC and between 75% and 100% for NAL, while the maximum standard deviation (SD) for the positive control replicates should be < 10% for both NAC and NAL depletion, and

c) the mean NAC and NAL concentration of both Reference Controls A and Reference Control C should be 3.2–4.4 μM and the coefficient of variation (CV) of NAC and NAL peak areas for the nine Reference Controls B and C in acetonitrile should be < 10%.

If one or more of these criteria is not satisfied, the data should be rejected and the run should be repeated.

59. The following criteria should be satisfied for a test chemical's results to be accepted as valid:

a) the maximum standard deviation for the test chemical replicates should be < 10% for the percent depletion of both NAC and NAL,

b) the mean NAC and NAL concentration of the three Reference Controls C in the appropriate solvent should be 3.2–4.4 μM.

If one or more of these criteria is not satisfied, the data should be rejected and the run should be repeated.

Prediction model

60. The mean percent depletion of NAC and NAL is calculated for each test chemical. Negative depletion is considered to be "0" when calculating the mean. By using the NAC/NAL prediction model shown in Table 1, the threshold of 4.9% mean percent depletion should be used to support the discrimination between skin sensitisers and non-sensitiser in the framework of an IATA or a DA.

Table 1: NAC/NAL prediction model[1]

Mean NAC and NAL percent depletion	ADRA prediction[2]
Less than 4.9%	Negative
4.9% or higher	Positive

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.
[2] An ADRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 3.

61. Co-elution occurs when the test chemical (the substance or one or several of the constituents of a multi-constituent substance or a mixture) absorbs significantly at 281 nm and has the same retention time as NAC or NAL. Co-elution may be resolved by slightly adjusting the HPLC set-up in order to further separate the elution time of the test chemical and NAC or NAL. If an alternative HPLC set-up is used to try to resolve co-elution, its equivalence to the validated set-up should be demonstrated, preferably by testing the proficiency substances in Annex 1. When co-elution occurs, it is not possible to integrate the peak of the NAC or NAL, thereby preventing calculation of the percent depletion of NAC or NAL. If co-elution of test chemicals occurs with both the NAC and NAL and separation of elution time is not feasible, then the analysis should be reported to be inconclusive. In cases where co-elution occurs only with NAL and separation of elution time is not feasible, the NAC-only prediction model (See Table 2) can be used to make a prediction.

Table 2: NAC-only prediction model[1]

Mean NAC percent depletion	ADRA prediction[2]
Less than 5.6%	Negative
5.6% or higher	Positive

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.
[2] An ADRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 3.

62. When a result is unequivocal, a single HPLC analysis for both NAC and NAL should be sufficient for a test chemical. Additional testing is sometimes necessary, however, when the results lie close to the threshold value used to discriminate between positive and negative results (borderline results). If the mean percent depletion falls between 3.0% and 10.0% when using the NAC/NAL prediction model or the NAC percent depletion falls between 4.0% and 11.0% when using the NAC-only prediction model, a second run is advisable, as is a third run in the event of discordant results between the first two runs.

Test report

63. The test report should include the following information:

Test chemical

- Mono-constituent substance
    - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers
    - Physical appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available
    - Purity, chemical identity of impurities as appropriate and practically feasible, etc.
    - Treatment prior to testing, if applicable (warming, grinding)
    - Concentration(s) tested
    - Storage conditions and stability to the extent available
- Multi-constituent substance, UVCB, and mixtures
    - Characterisation by chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available
    - Physical appearance, water solubility, and additional relevant physicochemical properties, to the extent available
    - Molecular weight (or apparent molecular weight) for mixtures or polymers of known composition, or other information relevant to the study
    - Treatment prior to testing, if applicable (warming, grinding)
    - Concentration(s) tested
    - Storage conditions and stability, to the extent available.

Controls

- Positive control
    - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
    - Physical appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
    - Purity, chemical identity of impurities, as appropriate or feasible
    - Treatment prior to testing, if applicable (warming, grinding)
    - Concentration(s) tested
    - Storage conditions and stability, to the extent available;
    - Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.

- Solvent
    - Solvent used and ratio of its constituents, if applicable
    - Chemical identification(s), such as IUPAC or CAS name(s), CAS number(s), and/or other identifiers
    - Purity, chemical identity of impurities, as appropriate and feasible
    - Physical appearance, molecular weight, and additional relevant physicochemical properties when solvents other than those mentioned in the test method are used
    - Storage conditions and stability, to the extent available
    - Justification for choice of solvent for each test chemical
    - Impact on NAC and NAL stability when using acetonitrile

Preparation of NAC and NAL, positive control and test chemical solution

- Characterisation of NAC and NAL solutions (supplier, lot, exact weight of NAC and NAL, volume added for the stock solution)
- Characterisation of positive control solutions (exact weight of positive control reagent, volume added for the control solution)
- Characterisation of test chemical solutions (exact weight of test chemical, volume added for the test chemical solution)

HPLC instrument setting and analysis

- Type of HPLC instrument, HPLC and guard columns, detector, autosampler
- Parameters relevant for the HPLC analysis such as column temperature, injection volumes, flow rate and gradient

System suitability

- NAC and NAL peak area at 281 nm of each standard and reference control A replicate
- Linear calibration curve graphically represented and the R2 reported
- NAC and NAL concentration of each Reference Control A replicate
- Mean NAC and NAL concentration (µM) of the three reference controls A, SD and CV
- NAC and NAL concentration of Reference Controls A and C.

Analysis sequence

- For Reference Controls
    - NAC and NAL peak area at 281 nm of each replicate of Reference Controls B and C
    - Mean NAC and NAL peak area at 281 nm of the nine Reference Controls B and C in acetonitrile, SD and CV (for stability of reference controls over analysis time)

- For each solvent used, the mean NAC and NAL peak area at 281 nm of the three appropriate Reference Controls C (for the calculation of percent NAC and NAL depletion)
- For each solvent used, the NAC and NAL concentration (μM) of the three appropriate Reference Controls C
- For each solvent used, the mean NAC and NAL concentration (μM) of the three appropriate Reference Controls C, SD and CV.

- For positive controls
  - NAC and NAL peak area at 281 nm of each replicate
  - Percent NAC and NAL depletion of each replicate
  - Mean percent NAC and NAL depletion of the three replicates, SD and CV.

- For each test chemical
  - Appearance of precipitate in the reaction mixture at the end of the incubation time, if observed. If precipitate was re-solubilised or centrifuged;
  - Presence of co-elution
  - Description of any other relevant observations, if applicable
  - NAC and NAL peak area at 281 nm of each replicate
  - Percent NAC and NAL depletion of each replicate
  - Mean of percent NAC and NAL depletion of the three replicate, SD and CV
  - Mean of percent NAC and percent NAL depletion values
  - Prediction model used and ADRA prediction

Proficiency testing

- If applicable, the procedure used to demonstrate proficiency of the laboratory in performing the test method (testing of proficiency substances, etc.) or to demonstrate reproducible performance of the test method over time.

Discussion of the results

- Discussion of the results obtained with the ADRA test method
- Discussion of the test method results in the context of an IATA if other relevant information is available

Conclusion

Literature for Appendix II

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APPENDIX II, ANNEX 1

Proficiency Substances

In Chemico Skin Sensitisation: Amino acid Derivative Reactivity Assay (ADRA)

Prior to routine use of the test method, laboratories should demonstrate technical proficiency by correctly obtaining the expected ADRA prediction for the 10 proficiency substances recommended in Table 1 and by obtaining NAC and NAL depletion values that fall within the respective reference ranges for 8 out of the 10 proficiency substances. These proficiency substances were selected to represent the full range of responses for skin sensitisation hazards. Other selection criteria were that they are commercially available, that high quality *in vivo* reference data and high quality ADRA data are available, and that they were used during the JaCVAM-coordinated validation study to demonstrate successful implementation.

Table 1. Recommended chemicals for demonstrating technical proficiency with ADRA

No.	Test chemicals	CAS No.	Physical state	Molecular weight	*In vivo* Prediction[1]	ADRA prediction[2]	Range of % depletion	
							NAC[3]	NAL[3]
1	*p*-Benzoquinone	106-51-4	Solid	108.09	Sensitiser (extreme)	Positive	90-100	40-70
2	Chloramine T trihydrate	7080-50-4	Solid	281.69	Sensitiser (strong)	Positive	90-100	90-100
3	Trans-Cinnamaldehyde	14371-10-9	Liquid	132.16	Sensitiser (moderate)	Positive	40-100	≤20
4	Palmitoyl Chloride	112-67-4	Liquid	274.87	Sensitiser (moderate)	Positive	≤10	50-100
5	Imidazolidinyl urea	39236-46-9	Solid	388.29	Sensitiser (weak)	Positive	10-45	≤10
6	Farnesal	19317-11-4	Liquid	220.35	Sensitiser (weak)	Positive	20-40	≤15
7	Glycerol	56-81-5	Liquid	92.09	Non-sensitiser	Negative	≤7	≤7
8	Benzyl alcohol	100-51-6	Liquid	108.14	Non-sensitiser	Negative	≤7	≤7
9	Dimethyl isophthalate	1459-93-4	Solid	194.19	Non-sensitiser	Negative	≤7	≤7
10	Propyl paraben	94-13-3	Solid	110.11	Non-sensitiser	Negative	≤7	≤7

[1]The *in vivo* hazard (and potency) predictions are based on LLNA data. (13) (14) (15). The *in vivo* potency is derived using the criteria proposed by ECETOC (16).

[2] An ADRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 3.

[3] Ranges determined on the basis of at least 10 depletion values generated by 5 independent laboratories.

APPENDIX II, ANNEX 2

EXAMPLES OF ANALYSIS SEQUENCE

Each sample of HPLC analysis should be analysed in number order below. Refer to the table showing Examples of HPLC Sample Analysis Sequences for more practical sequences about HPLC analysis.

1. Start to analyse calibration standards and Reference Control A (N = 3).

2. The co-elution Control does not need to be analysed by turns if it is analysed after analysis of standard solution and Reference Control A.

3. Reference Control B should be analysed three times (total six times) before and after the analysis of sample, Reference Control C and Positive Control.

4. The Reference Control C, Positive Control and Test chemical solutions are analysed. (After the first set of replicates of each sample is analysed, the second set of replicates of each should be analysed).

Calibration standards and reference controls	STD1 STD2 STD3 STD4 STD5 STD6 Dilution buffer Reference control A, rep 1 Reference control A, rep 2 Reference control A, rep 3
Co-elution controls	Co-elution control 1 for test chemical 1 Co-elution control 2 for test chemical 2
Reference controls	Reference control B, rep 1 Reference control B, rep 2 Reference control B, rep 3
First set of replicates	Reference control C, rep 1 Phenylacetaldehyde, rep 1 Sample 1, rep 1 Sample 2, rep 1
Second set of replicates	Reference control C, rep 2 Phenylacetaldehyde, rep 2 Sample 1, rep 2 Sample 2, rep 2
Third set of replicates	Reference control C, rep 3 Phenylacetaldehyde, rep 3 Sample 1, rep 3

	Sample 2, rep 3
Reference controls	Reference control B, rep 4 Reference control B, rep 5 Reference control B, rep 6

Three sets of reference controls (NAC or NAL dissolved in the appropriate solvent) should be included in the analysis sequence:

Reference control A: Control for verifying validity of the HPLC system. Reference Control A is used to verify concentration of NAC and NAL from each calibration curve after addition of acetonitrile rather than test chemical.

Reference control B: Control for verifying stability of reaction solution under analysis. Reference Control B is used to verify variability (CV) of each three NAC/NAL peak areas in the solution after addition of acetonitrile rather than test chemical at the start of analysis and at the end of analysis.

Reference control C:

Control for calculating NAC/NAL depletion of each test chemical solution. To calculate depletion of NAC/NAL, measure three Reference Controls C after addition of solvent instead of test chemical. Prepare reference Control C for all solvents used to dissolve the test chemicals.

Section 4
Health effects

Test Guideline No. 495

Reactive Oxygen Species (ROS) Assay for Photoreactivity

18 June 2019

OECD Guidelines for the Testing of Chemicals

OECD
BETTER POLICIES FOR BETTER LIVES

OECD GUIDELINE FOR TESTING OF CHEMICALS

Ros (Reactive Oxygen Species) Assay For Photoreactivity

INTRODUCTION

1. Phototoxicity is defined as a toxic response is elicited by topically or systemically administered photoreactive chemicals after the exposure of the body to environmental light. Several classes of photoreactive chemicals could cause phototoxic reactions when activated by light at otherwise non-toxic doses. Phototoxicity can be categorized as photoirritation, photoallergy, and photogenotoxicity (1). Photoirritation is characterized as an acute light-induced skin response to a photoreactive chemical. Photoallergy is an immune-mediated reaction in which light may cause a structural change in a drug so that it acts as a hapten, possibly by binding to proteins in the skin (2). Photogenotoxicity is a genotoxic response after exposure to a chemical by two mechanisms: either directly by photoexcitation of DNA or indirectly by excitation of photoreactive chemicals.

2. In 2002, regulatory agencies in the US (US Food and Drug Administration, FDA) and EU (European Medicines Agency, EMA) published guidelines for photosafety assessments of drug candidates (3)(4). In 2004, the Organisation for Economic Co-operation and Development (OECD) adopted Test Guideline 432: *In vitro* 3T3 Neutral Red Uptake (NRU) Phototoxicity Test as a validated methodology for evaluating the phototoxic potential of chemicals (5). The EMA also published a concept paper in 2008 (6), which proposes a testing strategy that merges the testing proposals recommended by FDA and EMA. Considering these documents, the International Council of Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) published ICH S10 guideline, "Photosafety Evaluation of Pharmaceuticals" in 2014 (7).

3. According to above referenced guidelines, chemicals or drug candidates need to be examined for their phototoxic potential. Since light must be absorbed by a compound in order for photochemical reactions to take place (8), the phototoxic potential of chemicals is related to the photochemical properties of compounds, especially light absorption properties within 290–700 nm. The guidelines suggested the need for measurement of the light absorption properties of chemicals as a first round of screening (3)(4). The ICH S10 guideline recommends UV-visible light absorption spectral analysis as a criterion for evaluating the phototoxic potentials of drugs (7); however, UV-visible light absorption of chemicals would not always correlate directly with their phototoxic potential, so a

combination of UV data (molar extinction coefficient, MEC) with other appropriate screening systems might be advantageous in avoiding false predictions.

4. In addition to light absorption and distribution to light-exposed tissue, the generation of a reactive species from chemicals following absorption of UV-visible light is described as a key determinant of chemicals for causing direct phototoxic reactions in an older guidance document (7)(9). Thus, the Reactive Oxygen Species (ROS) assay (10)(11) has been also included by the ICH S10 guideline as an optional initial *in chemico* screening tool for evaluating the photoreactivity of pharmaceuticals (7).

5. As an alternative method for *in vivo* phototoxicity testing, the OECD TG432 (5) describes an *in vitro* 3T3 NRU Phototoxicity Test and sets specific criteria for evaluating phototoxic hazard. The 3T3 NRU Phototoxicity Test evaluates photo-cytotoxicity by the relative reduction in viability of cells exposed to the chemical in the presence versus absence of light. Chemicals identified by this test are likely to be photoreactive, following systemic application and distribution to the skin, or after topical application. Although most of the photoirritant chemicals were correctly identified by the 3T3 NRU Phototoxicity Test, it provided false predictions for almost half of the chemicals in the photoallergens group. However, the 3T3 NRU Phototoxicity Test was not originally designed for specific prediction of chemical photoallergenicity (2). The 3T3 NRU Phototoxicity Test seems to be less reliable for photoallergenicity prediction. The photochemical assays such as ROS assay and UV/VIS spectral analysis can predict photoallergenic potential of tested chemicals, although there is still a substantial risk of false positive predictions (12).

6. Definitions used are provided in Annex A.

INITIAL CONSIDERATION AND LIMITATIONS

7. Before photosafety assessments are considered, a UV-visible light absorption spectrum of the test chemical should be determined according to OECD Test Guideline 101(13). Based on an analysis of data, the ICH S10 guideline has suggested that no further photosafety testing is needed if the MEC of a chemical is less than 1,000 $L \cdot mol^{-1} \cdot cm^{-1}$ (7). Few phototoxic chemicals showed a MEC less than 1,000 $L \cdot mol^{-1} \cdot cm^{-1}$ and these chemicals may not need to be tested in the ROS assay or any other photosafety assessments (9) (14) (15). Data collected for the limits of photoreactivity are discussed in Henry *et al.* (16) and Bauer *et al.* (17). It should be noted that phototoxicity by indirect mechanisms (e.g., pseudoporphyria or porphyria), although rare, could still occur. For compounds with MEC values of 1000 $L \cdot mol^{-1} \cdot cm^{-1}$ or higher, if the drug developer chooses to conduct a test for photoreactivity a negative result could support a decision that no further photosafety assessment is warranted.

8. The reliability and relevance of the ROS assay was recently evaluated in a multi-laboratory validation study using two different solar simulators (18)(19)(20)(21). In both solar simulators, the intra- and inter-day precisions for quinine, a positive control, were found to be above 90%, and the data suggested high inter-laboratory reproducibility (19). In a multi-laboratory validation study, the ROS assay on 2 standards and 42 coded chemicals, including 23 phototoxins and 19 non-phototoxic drugs/chemicals, provided no false negative predictions upon defined criteria as compared with the *in vitro/in vivo* phototoxicity. The, sensitivity, individual specificity, positive and negative predictivities of the ROS assay on the 42 tested chemical were calculated to be 100%, 42–82%, 75–92% and 100%, respectively. The ROS assay was designed for qualitative photoreactivity assessment of chemicals, the principle of which is

monitoring of type I (an electron or hydrogen transfer, resulting in the formation of free radical species) and type II (an energy transfer from excited triplet photosensitizer to the oxygen) photochemical reactions in test chemicals exposed to simulated sunlight (10), possibly leading to photodegradation and various phototoxic reactions, including photoirritation, photoallergy, and photogenotoxicity. Further, this assay has been optimised for detecting positive test chemicals. Test chemicals found to be negative in the ROS assay are likely to be negative in *in vivo* test systems; however, additional data may be required to determine if chemicals that are photoreactive in the ROS assay are likely to be positive *in vivo*. The test has not been designed to address indirect mechanisms of phototoxicity, such as effects of metabolites of a test chemical.

9. The applicability domain of the ROS assay is currently restricted to only those chemicals that meet the solubility criteria outlined in the protocol (see paragraph 22). Insoluble chemicals in the reaction mixtures are not suitable for testing with the ROS assay using this protocol (DMSO or NaPB solvent) but might be tested in the ROS assay with addition of solubility enhancers in the reaction mixtures (22)(23)(24). However, further characterization and standardization of procedures using these alternative vehicles should be performed by testing proficiency chemicals before incorporation into routine use. In the ROS assay, superoxide anion (SA) can be measured upon the reduction of nitroblue tetrazolium, and the determination of singlet oxygen (SO) can be made on the basis of bleaching of *p*-nitrosodimethylaniline by oxidized imidazole (11). Test chemicals that interfere with these reactions are sometimes best considered outside of the applicability domain of the ROS assay. For example, ascorbic acid and other reducing chemicals reduce the tetrazolium salt to formazan directly (25). Some skin-lightening cosmetics may also have potent reducing properties that interfere with ROS determinations. Ascorbic acid also accelerates the oxidation of imidazole derivatives (26), providing false positive prediction in the ROS assay.

10. The term "test chemical" is used in this Test Guideline to refer to what is being tested and is not related to the applicability of the ROS assay to the testing of mono-constituent chemicals, multi-constituent chemicals and/or mixtures. Based on the data currently available, the ROS assay was shown to be applicable to test chemicals covering a variety of organic functional groups, reaction mechanisms, phototoxic potency (as determined in *in vivo* studies) and physicochemical properties. Limited information is currently available on the applicability of the ROS assay to multi-constituent chemicals/mixtures (27). When considering testing of mixtures, difficult to test chemical (e.g. unstable) or chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically.

PRINCIPLE OF THE TEST

11. Chemical phototoxicity can be caused by topical and systemic application of chemicals in combination with exposure to environmental light. There are several classes of chemicals that are nontoxic by themselves but could become reactive in the skin or eyes when exposed to environmental light and thereby result in toxicity. The primary event in any phototoxic reaction is the absorption of photons of a wavelength that induces excitation of the chromophore. The excitation energy is often transferred to oxygen molecules, followed by generation of ROS, including SA through type I photochemical reactions and SO through type II photochemical reactions by photo-excited molecules. These appear to be the principal intermediate species in many phototoxic responses. Direct reaction of excited chromophores with cellular constituents may also lead to phototoxicity. Therefore, while the ROS assay may not detect all ultimate mechanisms of phototoxicity, the

determination of ROS generation from chemicals irradiated with simulated sunlight is indicative of phototoxic potential.

12. In the ROS assay, SO generation is detected by spectrophotometric measurement of *p*-nitrosodimethylaniline (RNO) bleaching, followed by decreased absorbance of RNO at 440 nm (28). Although SO does not react chemically with RNO, the RNO bleaching is a consequence of SO capture by the imidazole ring, which results in the formation of a trans-annular peroxide intermediate capable of inducing the bleaching of RNO, as follows:

SO + Imidazole → [Peroxide intermediate] → Oxidized imidazole

[Peroxide intermediate] + RNO → RNO + Products

13. SA generation is detected by observing the reduction of nitroblue tetrazolium (NBT). As indicated below; NBT can be reduced by SA via a one-electron transfer reaction, yielding partially reduced (2 e^-) monoformazan (NBT^+) as a stable intermediate (29). Thus, SA can reduce NBT to NBT^+, the formation of which can be monitored spectrophotometrically at 560 nm.

$SA + NBT \rightarrow O_2 + NBT^+$

Demonstration of Proficiency

14. Prior to routine use of the test method described in this Test Guideline, laboratories should demonstrate technical proficiency, using the proficiency chemicals listed and described in Annex C. The 9 proficiency chemicals (Nos. 1–9) for the two recommended solar simulators (Suntest CPS+ or CPS and SXL-2500V2) or the 17 proficiency chemicals (Nos. 1–17) for a solar simulator other than the two recommended models are to be tested to ensure that measured values of SO and SA on all proficiency chemicals are within the range described in Annex C.

PROCEDURE

Solar simulator

15. Typically calibrated solar simulators are used because photoreactivity in the presence of natural sunlight is of concern, due to the spectral differences of global positioning and the time of day. For other circumstances where photoreactivity in response to artificial light is of interest, other sources of light may be considered. An appropriate solar simulator is to be used for irradiation of UV and visible light. The irradiation power distribution is to be kept as close to that of outdoor daylight as possible by using an appropriate filter to reduce UVC wavelengths. Recommended test conditions are as follows:

Solar simulator with filter to reduce UV wavelengths <290 nm (See Annex B)

- 1.8 to 2.2 mW/cm^2 (e.g. the indicator setting value of 250 W/m^2 for CPS+) for 1 hour,
- 6.5 to 7.9 J/cm^2 of UVA intensity (Annex B).

SXL-2500V2 (Seric) with UV filter (to reduce wavelengths <300 nm)

- 3.0 to 5.0 mW/cm^2 for 1 hour,
- 11 to 18 J/cm^2 of UVA intensity (Annex B).

16. The solar simulator is to be equipped with an appropriate temperature control or fan to stabilize the temperature during irradiation, because ROS production is affected by

temperature. Standard temperature for a solar simulator with temperature control is 25°C. The acceptable temperature range during irradiation is 20 to 29°C (20)(21).

Quartz reaction container

17. A quartz reaction container is used to avoid loss of UV due to passing through a plastic lid and vaporization of the reaction mixture (20)(21)(30). Specifications for the recommended container are provided in Annex D. If a different container is used, a lid or seal with high UV transmittance should be used. In this case, a feasibility study using the reference chemicals (Nos. 1–17) is to be conducted to determine an appropriate level of exposure to UV and visible light.

Reagents

18. All reagents should be used within 1 month after preparation and should be sonicated immediately prior to use (20)(21). Representative preparation methods are shown as follows:

20 mM sodium phosphate buffer (NaPB), pH 7.4

- Weigh 593 mg of $NaH_2PO_4 \cdot 2H_2O$ (CAS No. 13472-35-0) and 5.8 g of $Na_2HPO_4 \cdot 12H_2O$ (CAS No. 10039-32-4), add 900 mL of purified water, adjust with HCl to a pH of 7.4, dilute with purified water up to 1 L, and mix.
- Store in a refrigerator or at room temperature.

0.2 mM *p*-nitrosodimethylaniline (RNO, CAS No. 138-89-6)

- Dissolve 3 mg of RNO in 100 mL of 20 mM NaPB.
- Store in a refrigerator and protect from light.

0.2 mM imidazole (CAS No. 288-32-4)

- Dissolve 13.6 mg of imidazole in 10 mL of 20 mM NaPB.
- Dilute the 20 mM imidazole solution 100 times with 20 mM NaPB.
- Store in a refrigerator and protect from light.

0.4 mM nitroblue tetrazolium chloride (NBT, CAS No. 298-83-9)

- Dissolve 32.7 mg of NBT in 100 mL of 20 mM NaPB.
- Store in a refrigerator and protect from light.

Solvents

19. Use analytical grade DMSO at first. For chemicals that are not soluble in DMSO, 20 mM NaPB is to be used as a solvent. Some chemicals react with DMSO and test chemical stability in DMSO should be determined. If the test chemical is not soluble or stable in DMSO or NaPB, other solvents may be used. However the test chemical must be demonstrated to be stable in the selected solvent, and SO and SA ranges for proficiency chemicals must fall within the ranges defined in Annex C.

Test chemicals

20. Test chemicals must be prepared fresh, immediately prior to use unless data demonstrate their stability in storage. It is recommended that all chemical handling and the initial treatment of cells be performed under light conditions that would avoid photoactivation or degradation of the test chemical prior to irradiation. Chemicals should be tested at 200 µM (final concentration). A 20-µM concentration can be used if precipitation occurs before light exposure, coloration, or other interference is observed in the reaction mixture at 200 µM. A positive result at 20 µM can be used to indicate

photoreactivity; however, a negative result at the lower 20 µM concentration is not indicative of absence of photoreactivity. The molecular weight of the test chemical must be available.

21. The test chemical solutions are to be prepared immediately before use in a solvent as described in paragraph 19. Each test chemical is to be weighed in a tube, and solvent added to achieve a 10 mM concentration of the test chemical (20)(21). The tube is to be mixed with a vortex mixer and sonicated for 5 to 10 minutes. All preparations are to be protected from strong UV and intense visible light (e.g. direct overhead light, working near windows exposed to natural light) at all times during preparation. When precipitation before light exposure or other interference is observed in the reaction mixture at 200 µM, a 1-mM solution (20 µM as the final concentration) is to be prepared by dilution of the stock solution of chemicals at 10 mM using DMSO. For chemicals that are not soluble in DMSO, 20 µL of DMSO (2 v/v%) is to be contained in the reaction mixture.

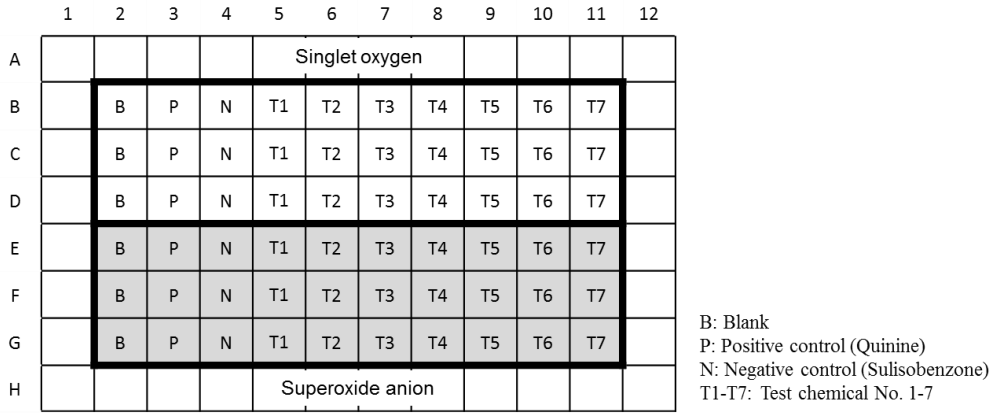
Positive and negative controls

22. Stock solutions of quinine hydrochloride (a positive control, CAS No. 6119-47-7) and sulisobenzone (a negative control, CAS No. 4065-45-6) are to be prepared at 10 mM each in DMSO (final concentration of 200 µM) according to the above procedure, divided into tubes, and stored in a freezer (generally below -20°C) for up to 1 month. The stock solution is to be thawed just before the experiment and used within the day.

Test procedure

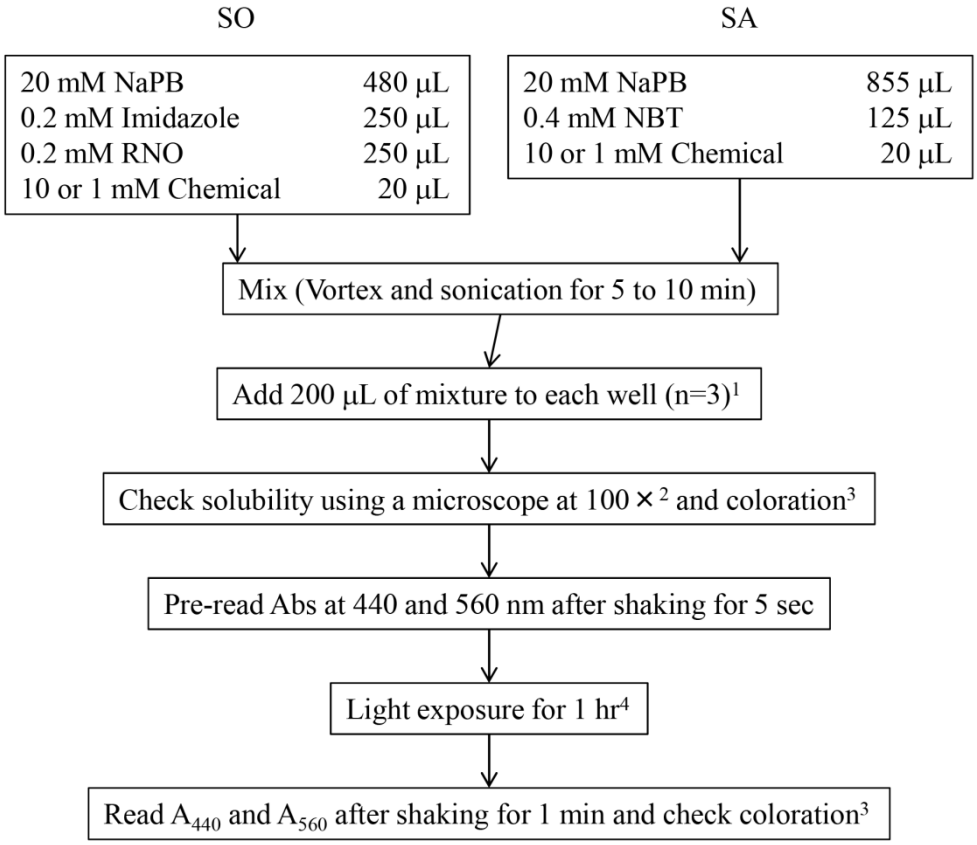
23. A typical 96-well plate configuration is as follows, but other configurations are also acceptable:

Figure 1. An example of a typical plate configuration



24. A tube (e.g. 1.5 mL micro tube) and a plastic clear flat bottomed 96-well microplate are to be used. The reaction mixture is to be prepared by vortex mixing and/or sonication under UV-cut illumination or shade. The same volume of DMSO, 20 µL, is to be added in a vehicle control instead of test chemical solution.

Figure 2. Workflow diagram if the stock solution of the test chemical is prepared in DMSO.



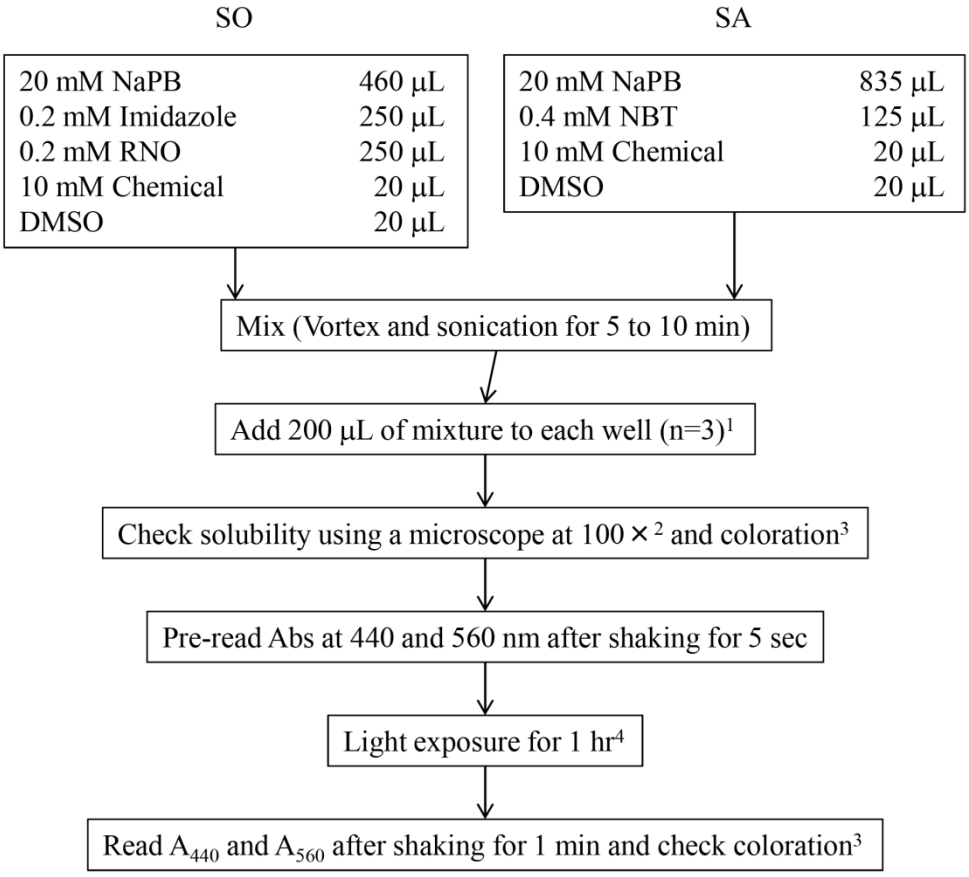
[1] Avoid using peripheral wells. More than one test chemical can be tested on a plate.

[2] Some chemicals might precipitate in the reaction mixture. It is therefore important to check solubility prior to irradiation. Solubility of each reaction mixture in its well is to be observed with a microscope prior to irradiation. Test chemical concentrations are to be selected so as to avoid precipitation or cloudy solutions.

[3] The reaction mixture is to be checked for coloration with the naked eye.

[4] The 96-well plate is to be placed in the quartz reaction container. A quartz cover is to be set on the plate and fastened with bolts. Ensure that temperature and other ambient conditions are stable when using the solar simulator. Measure UVA intensity and temperature at the plate position using a UVA detector and thermometer both before and after irradiation.

Figure 3. Workflow diagram if the stock solution of the test chemical is prepared in 20 mM NaPB.



[1] Avoid using peripheral wells. More than one test chemical can be tested on a plate.

[2] Some chemicals might precipitate in the reaction mixture. It is therefore important to check solubility prior to irradiation. Solubility of each reaction mixture in its well is to be observed with a microscope prior to irradiation. Test chemical concentrations are to be selected so as to avoid precipitation or cloudy solutions.

[3] The reaction mixture is to be checked for coloration with the naked eye.

[4] The 96-well plate is to be placed in the quartz reaction container. A quartz cover is to be set on the plate and fastened with bolts. Ensure that temperature and other ambient conditions are stable when using the solar simulator. Measure UVA intensity and temperature at the plate position using a UVA detector and thermometer both before and after irradiation.

DATA AND REPORTING

Data analysis

25. Data from three wells for each chemical concentration is used to calculate mean and standard deviation.

SO

Decrease of A_{440} ×1000 = [A_{440} (–) – A_{440} (+) – (a – b)] ×1000
A_{440} (–): Absorbance before light exposure at 440 nm
A_{440} (+): Absorbance after light exposure at 440 nm
a: Vehicle control before light exposure (mean)
b: Vehicle control after exposure (mean)

SA

Increase of A_{560} ×1000 = [A_{560} (+) – A_{560} (–) – (b – a)] ×1000
A_{560} (–): Absorbance before light exposure at 560 nm
A_{560} (+): Absorbance after light exposure at 560 nm
a: Vehicle control before light exposure (mean)
b: Vehicle control after exposure (mean)

Criteria for data acceptance

26. The following criteria are to be satisfied in each experiment.

- No precipitation of test chemical in the reaction mixture before light exposure.
- No color interference by test chemical in the reaction mixture before or after light exposure.
- No technical problems, including temperature range (20–29°C), when collecting data set.
- The ranges of raw A_{440} and A_{560} values: 0.02 to 1.5.
- Historical positive and negative control values are to be developed by each laboratory based on a mean +/-2 SD. The following range was defined based on the 95% confidence interval (mean +/- 1.96SD) obtained from the validation data. When a solar simulator other than a recommended model is used, establish modified criteria based on 95% confidence interval.
-

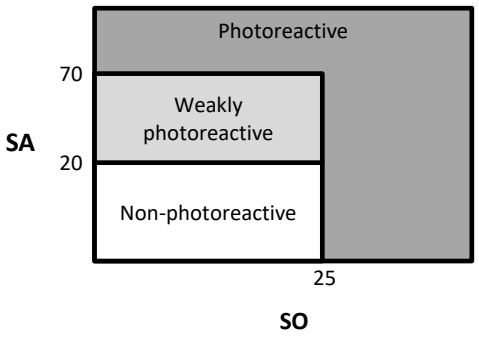
  Positive control (quinine hydrochloride) value at 200 µM (mean of 3 wells)
  SO: 319 to 583
  SA: 193 to 385
  Negative control (sulisobenzone) value at 200 µM (mean of 3 wells)
  SO: -9 to 11
  SA: -20 to 2
- Laboratories should demonstrate technical proficiency, as described in Annex C, prior to routine use of the test method described in this Test Guideline.

Criteria for judgment

27. Each test chemical is to be judged as follows:

ROS assay prediction model

Judgment[1, 2]	Concentration[3]	SO (mean of 3 wells)[6]		SA (mean of 3 wells)[6]
Photoreactive	200 μM	≥25	and	≥70
		<25 and/or I[4]	and	≥70
		≥25	and	<70 and/or I[4]
Weakly photoreactive	200 μM	<25	and	≥20, <70
Photoreactive	20 μM	≥25	and	≥20
Non-photoreactive	200 μM	<25	and	<20
Inconclusive	The results do not meet any of the above-mentioned criteria.[5]			



[1] A single experiment is sufficient for judging results, because the ROS assay shows good intra- and inter-laboratory reproducibility in the validation studies.

[2] If precipitation, coloration, or other interference is observed at both 20 and 200 μM, the chemical is considered incompatible with the ROS assay and judged as inconclusive.

[3] 20 μM can be used for judgment when precipitation or coloration is observed at 200 μM. A positive results at 20 μM can be used to indicate photoreactivity; however, a negative result at the lower 20 μM concentration is not indicative of absence of photoreactivity.

[4] Interference such as precipitation or coloration.

[5] Positive prediction can be made on the basis of SO only, SA only, or both; however, both SO and SA values should be obtained for reliable negative prediction.

[6] Classification criteria defined in published manuscripts. (11)(20)(21)

Data quality

28. Studies for regulatory purposes are to be conducted to the highest of quality standards, with data collection records readily available, in compliance with GLP regulations whenever possible, and all documents checked by the Quality Assurance Unit of the laboratory.

Test report

29. The test report should include the following information:

Test chemical:

- identification data, common generic names and IUPAC and CAS number, if known;
- physical nature and purity;
- physicochemical properties relevant to conduct of the study;
- UV/vis absorption spectrum;
- stability and photostability, if known.

Control chemicals:

- name, manufacturer, and lot No.;
- physical nature and purity;
- storage condition;
- preparation of control chemical solutions;
- final concentrations tested.

Solvent:

- name, manufacturer, and lot No.;
- justification for choice of solvent;
- solubility of the test chemical in solvent.

Irradiation condition:

- manufacturer and type of the solar simulator used;
- rationale for selection of the solar simulator used;
- UVA detector used;
- UVA irradiance, expressed in mW/cm^2
- UVA dose, expressed in J/cm^2;
- temperature before and after irradiation.

ROS assay procedure.

Acceptance and decision criteria.

Results.

Discussion.

Conclusions.

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Annex A. Definitions

3T3 NRU Phototoxicity Test: *In vitro* 3T3 neutral red uptake phototoxicity test.

Irradiance: The intensity of UV or visible light incident on a surface, measured in W/m^2 or mW/cm^2.

Dose of light: The quantity [= intensity × time (seconds)] of UV or visible light incident on a surface, expressed in J/m^2 or J/cm^2.

MEC: Molar Extinction Coefficient (also called molar absorptivity) is a constant for any given molecule under a specific set of conditions (e.g. solvent, temperature, and wavelength) and reflects the efficiency with which a molecule can absorb a photon (typically expressed as $L \cdot mol^{-1} \cdot cm^{-1}$).

Photoreactivity: The property of chemicals that react with another molecule as a consequence of absorption of photons.

Phototoxicity: Toxic responses that can be elicited after the exposure of skin to certain chemicals and subsequent exposure to light, or that is induced similarly by skin irradiation after systemic administration of a chemical.

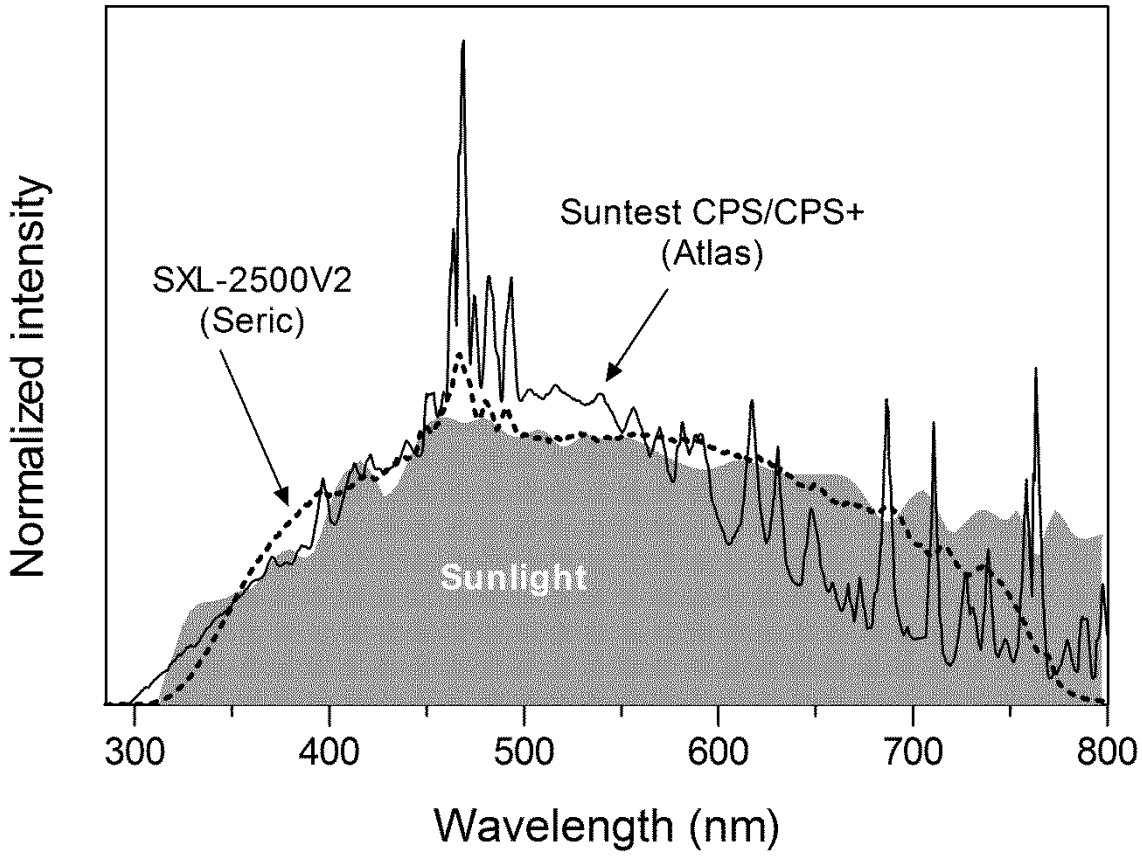
ROS: Reactive Oxygen Species, including superoxide anion (SA) and singlet oxygen (SO).

SA: Superoxide anion is one of radical species, generated from photo-irradiated chemicals through type I photochemical reaction.

SO: Singlet oxygen is one of radical species, generated from photo-irradiated chemicals through type II photochemical reaction.

UV light wavebands: The designations recommended by the CIE (Commission Internationale de L'Eclairage) are: UVA (315–400 nm) UVB (280–315 nm) and UVC (100–280 nm). Other designations are also used; the division between UVB and UVA is often placed at 320 nm, and the UVA may be divided into UV-A1 and UV-A2 with a division made at about 340 nm.

Annex B. Spectrum of solar stimulators used in the validation studies.



Annex C. Proficiency Chemicals

Prior to routine use of the test method described in this Test Guideline, laboratories should demonstrate technical proficiency by correctly obtaining the expected ROS prediction for proficiency chemicals recommended in the Table. For Suntest CPS/CPS+ (Atlas) or SXL-2500V2 (Seric) solar simulators, nine chemicals (Nos. 1–9) are to be tested. For other solar simulators, all 17 chemicals (Nos. 1–17) are to be tested. These proficiency chemicals were selected to represent the range of responses for phototoxic potential. Other selection criteria were that they are commercially available, that high quality *in vivo* reference data and high quality *in vitro* data generated with the ROS assay are available, and that they were used in the JaCVAM-coordinated validation study to demonstrate successful implementation of the test method in the laboratories participating in the study (20)(21).

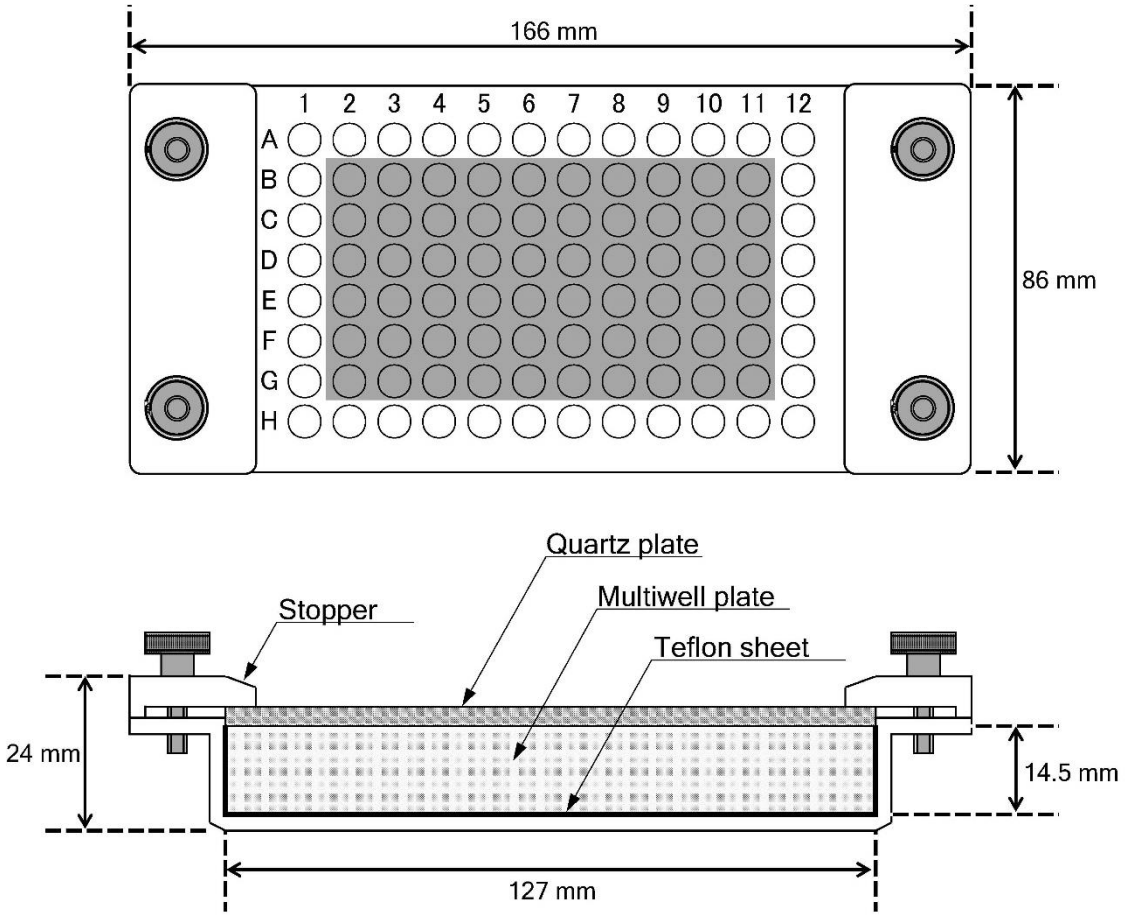
Table A C.1. Table of proficiency chemicals.

The expected ROS prediction for proficiency chemicals and the acceptable range..

No.	Chemical[1]	CAS No.	SO [2]	SA [2]	Solvent	Concentration
1	*p*-Aminobenzoic acid	150-13-0	-8 to 12	-11 to 7	DMSO	200 µM
2	Benzocaine	94-09-7	-7 to 9	-7 to 17	DMSO	200 µM
3	Doxycycline hydrochloride	10592-13-9	115 to 429	230 to 468	DMSO	200 µM
4	Erythromycin	114-07-8	-15 to 11	-9 to 21	DMSO	200 µM
5	Fenofibrate	49562-28-9	77 to 203	-31 to 11	DMSO	20 µM
6	L-Histidine	71-00-1	-8 to 12	8 to 120	NaPB	200 µM
7	Norfloxacin	70458-96-7	131 to 271	57 to 161	DMSO	200 µM
8	8-Methoxy psoralen	298-81-7	31 to 137	0 to 126	DMSO	200 µM
9	Octyl salicylate	118-60-5	-5 to 11	-8 to 20	DMSO	20 µM
10	Acridine	260-94-6	182 to 328	121 to 243	DMSO	200 µM
11	Chlorpromazine hydrochloride	69-09-0	-56 to 70	66 to 106	DMSO	200 µM
12	Diclofenac	15307-79-6	34 to 416	47 to 437	DMSO	200 µM
13	Furosemide	54-31-9	31 to 225	-7 to 109	DMSO	200 µM
14	Ketoprofen	22071-15-4	120 to 346	77 to 151	DMSO	200 µM
15	Nalidixic acid	389-08-2	54 to 246	88 to 470	DMSO	200 µM
16	Omeprazole	73590-58-6	-221 to 103	30 to 216	DMSO	200 µM
17	Promethazine hydrochloride	58-33-3	20 to 168	-3 to 77	DMSO	200 µM

[1]All chemicals are solid
[2]The values were calculated as means +/- 1.96 SD from the validation data..

Annex D. Quartz reaction container used in the validation studies.

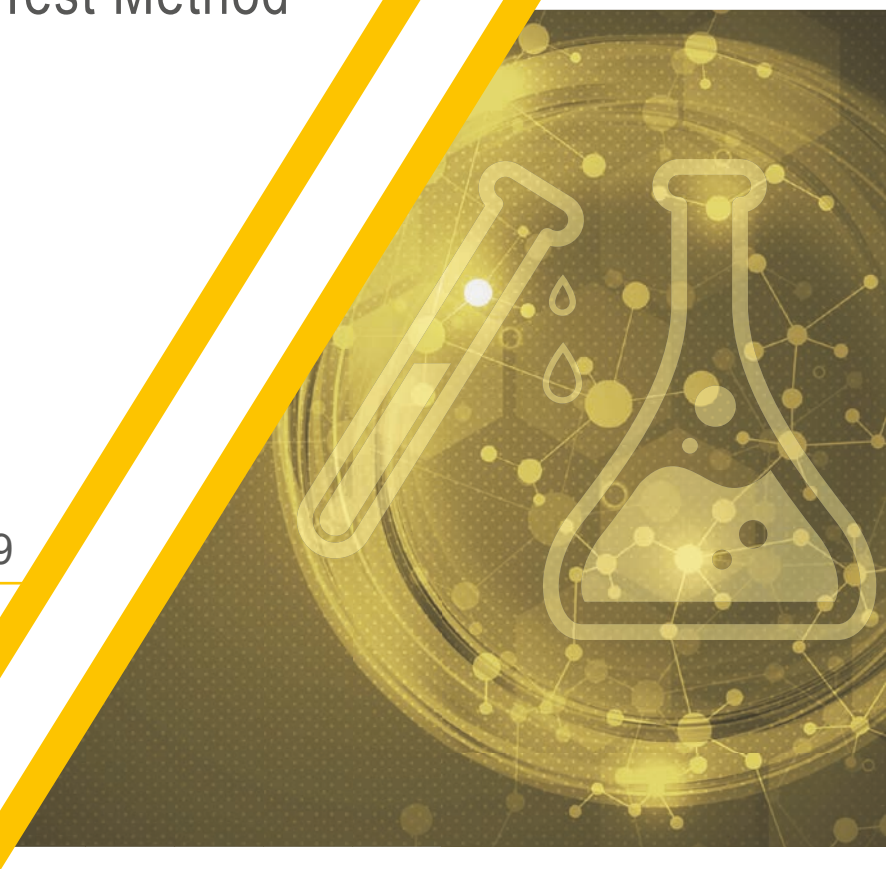


Recommended thickness of quartz plate: ca. 3 mm.

Section 4
Health effects

Test Guideline No. 431

In Vitro Skin Corrosion: Reconstructed Human Epidermis (RhE)Test Method



18 June 2019

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OECD GUIDELINE FOR TESTING OF CHEMICALS

In Vitro Skin Corrosion: Reconstructed Human Epidermis (RhE) Test Method

INTRODUCTION

1. Skin corrosion refers to the production of irreversible damage to the skin manifested as visible necrosis through the epidermis and into the dermis, following the application of a test chemical [as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS)] (1). This updated Test Guideline 431 provides an in vitro procedure allowing the identification of non-corrosive and corrosive substances and mixtures in accordance with UN GHS (1). It also allows a partial sub-categorisation of corrosives.

2. The assessment of skin corrosion potential of chemicals has typically involved the use of laboratory animals (OECD Test Guideline 404 (TG 404); originally adopted in 1981 and revised in 1992, 2002 and 2015) (2). In addition to the present TG 431, two other in vitro test methods for testing corrosion potential of chemicals have been validated and adopted as OECD Test Guidelines 430 (3) and 435 (4). Furthermore the in vitro OECD TG 439 (5) has been adopted for testing skin irritation potential. A document on Integrated Approaches to Testing and Assessment (IATA) for Skin Corrosion and Irritation describes several modules which group information sources and analysis tools, and provides guidance on (i) how to integrate and use existing testing and non-testing data for the assessment of skin irritation and skin corrosion potentials of chemicals and (ii) proposes an approach when further testing is needed (6).

3. This Test Guideline addresses the human health endpoint skin corrosion. It makes use of reconstructed human epidermis (RhE) (obtained from human derived non-transformed epidermal keratinocytes) which closely mimics the histological, morphological, biochemical and physiological properties of the upper parts of the human skin, i.e. the epidermis. This Test Guideline was originally adopted in 2004 and updated in 2013, 2016 and 2019 to include additional test methods using the RhE models. The Test Guideline was also updated in 2015 to introduce the possibility to use the methods to support the sub-categorisation of corrosive chemicals, and to refer to the IATA guidance document, and introduce the use of an alternative procedure to measure viability.

4. Five validated test methods using commercially available RhE models are included in this Test Guideline, as described below. Prevalidation studies (7), followed by a formal validation study for assessing skin corrosion (8) (9) (10) have been conducted (11) (12) for two of these commercially available test methods, EpiSkin™ Standard Model (SM), and EpiDerm™ Skin Corrosivity Test (SCT) (EPI-200) (referred to in the following text as the Validated Reference Methods – VRMs, EpiSkin^{TM}=VRM1, EpiDerm^{TM}= VRM2). The outcome of these studies led to the recommendation that the two VRMs mentioned above could be used for regulatory purposes for distinguishing corrosive (C) from non-corrosive (NC) substances, and that the EpiSkin™ could moreover be used to support sub-categorisation of corrosive substances (13) (14) (15). Two other commercially available in vitro skin corrosion RhE test methods have subsequently shown similar results to the EpiDerm™ SCT according to PS-based Validation (16) (17) (18). These are the SkinEthicTM RHE1 and epiCS® (previously named EST-1000) that can also be used for regulatory purposes for distinguishing corrosive from non-corrosive substances (19) (20). Post validation studies performed by the RhE model producers in the years 2012 to 2014 with a refined protocol correcting interferences of unspecific MTT reduction by the test chemicals improved the performance of both discrimination of C/NC as well as supporting sub-categorization of corrosives (21) (22). Further statistical analyses of the post-validation data generated with Epiderm™ SCT, SkinEthic™ RHE and epiCS® have been performed to identify alternative predictions models that improved the predictive capacity for sub-categorisation (23). Finally, the LabCyte EPI-MODEL24 is another commercially available in vitro skin corrosion RhE test that was shown to be scientific similar to the VRMs and can therefore be used for regulatory purposes to distinguish corrosive from non-corrosive substances as well as support sub-categorization of corrosives (40) (41) (42)(43).

5. Before a proposed similar or modified in vitro RhE test method for skin corrosion other than the VRMs can be used for regulatory purposes, its reliability, relevance (accuracy), and limitations for its proposed use should be determined to ensure its similarity to the VRMs, in accordance with the requirements of the Performance Standards (PS) (24) set out in accordance with the principles of Guidance Document No.34 (25). The Mutual Acceptance of Data will only be guaranteed after any proposed new or updated test method following the PS have been reviewed and included in this Test Guideline. The test methods included in this Test Guideline can be used to address countries' requirements for test results on in vitro test method for skin corrosion, while benefiting from the Mutual Acceptance of Data.

DEFINITIONS

6. Definitions used are provided in Annex I.

INITIAL CONSIDERATIONS

7. This Test Guideline allows the identification of non-corrosive and corrosive substances and mixtures in accordance with the UN GHS (1). This Test Guideline further supports the sub-categorisation of corrosive substances and mixtures into optional Sub-category 1A, in accordance with the UN GHS (1), as well as a combination of Sub-categories 1B and 1C (21) (22) (23). A limitation of this Test Guideline is that it does not allow discriminating between skin corrosive Sub-category 1B and Sub-category 1C in accordance with the UN GHS (1) due to the limited set of well-known in vivo corrosive Sub-category 1C chemicals. The five test methods under this test guideline are able to discriminate sub-categories 1A versus 1B-and-1C versus NC.

8. A wide range of chemicals representing mainly individual substances has been tested in the validation studies supporting the test methods included in this Test Guideline. The original database of the validation study conducted for identification of non-corrosives versus corrosives amounted to 60 chemicals covering a wide range of chemical classes (8) (9) (10). Testing to demonstrate sensitivity, specificity, accuracy and within-laboratory-reproducibility of the assay for sub-categorisation was further performed by the test method developers using 79 to 80 chemicals also covering a wide range of chemical classes, and results were reviewed by the OECD (21) (22) (23). On the basis of the overall data available, the Test Guideline is applicable to a wide range of chemical classes and physical states including liquids, semi-solids, solids and waxes. The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. Whenever possible, solids should be ground to a fine powder before application; no other prior treatment of the sample is required. In cases where evidence can be demonstrated on the non-applicability of test methods included in the Test Guideline to a specific category of test chemicals, these test methods should not be used for that specific category of test chemicals. In addition, this Test Guideline is assumed to be applicable to mixtures as an extension of its applicability to substances. However, due to the fact that mixtures cover a wide spectrum of categories and composition, and that only limited information is currently available on the testing of mixtures, in cases where evidence can be demonstrated on the non-applicability of the Test Guideline to a specific category of mixtures (e.g. following a strategy as proposed in (26)), the Test Guideline should not be used for that specific category of mixtures. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture. Gases and aerosols have not been assessed yet in validation studies (8) (9) (10). While it is conceivable that these can be tested using RhE technology, the current Test Guideline does not allow testing of gases and aerosols.

9. Test chemicals absorbing light in the same range as MTT formazan and test chemicals able to directly reduce the vital dye MTT (to MTT formazan) may interfere with the tissue viability measurements and need the use of adapted controls for corrections. The type of adapted controls that may be required will vary depending on the type of interference produced by the test chemical and the procedure used to measure MTT formazan (see paragraphs 25-31).

10. While this Test Guideline does not provide adequate information on skin irritation, it should be noted that OECD TG 439 specifically addresses the health effect skin irritation in vitro and is based on the same RhE test system, though using another protocol (5). For a full evaluation of local skin effects after a single dermal exposure, the Guidance Document No. 203 on Integrated Approaches for Testing Assessment should be consulted (6). This IATA approach includes the conduct of in vitro tests for skin corrosion (such as described in this Test Guideline) and skin irritation before considering testing in living animals. It is recognized that the use of human skin is subject to national and international ethical considerations and conditions.

PRINCIPLE OF THE TEST

11. The test chemical is applied topically to a three-dimensional RhE model, comprised of non-transformed, human-derived epidermal keratinocytes, which have been cultured to

form a multi-layered, highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multi-layered stratum corneum containing intercellular lamellar lipid layers representing main lipid classes analogous to those found in vivo.

12. The RhE test method is based on the premise that corrosive chemicals are able to penetrate the stratum corneum by diffusion or erosion, and are cytotoxic to the cells in the underlying layers. Cell viability is measured by enzymatic conversion of the vital dye MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue tetrazolium bromide; CAS number 298-93-1], into a blue formazan salt that is quantitatively measured after extraction from tissues (27). Corrosive chemicals are identified by their ability to decrease cell viability below defined threshold levels (see paragraphs 35 and 36). The RhE-based skin corrosion test methods have shown to be predictive of in vivo skin corrosion effects assessed in rabbits according to the OECD guideline 404 (2).

DEMONSTRATION OF PROFICIENCY

13. Prior to routine use of any of the five validated RhE test methods that adhere to this Test Guideline, laboratories should demonstrate technical proficiency by correctly classifying the twelve Proficiency Substances listed in Table 1. In case of the use of a method for sub-classification, also the correct sub-categorisation should be demonstrated. In situations where a listed substance is unavailable or where justifiable, another substance for which adequate in vivo and in vitro reference data are available may be used (e.g. from the list of reference chemicals (24)) provided that the same selection criteria as described in Table 1 are applied.

Table 1. List of Proficiency Substances[1]

Substance	CASRN	Chemical Class[2]	UN GHS Cat. Based on *In Vivo* results [3]	Cat. Based on *In Vitro* results[4]	Mean cell viability for VRMs				Physical State
					VRM1		VRM2		
					3 min	60 min.	3 min.	60 min	
Sub-category 1A *In Vivo* Corrosives									
Bromoacetic acid	79-08-3	Organic acid	1A	(3) 1A	3	2.8	3.2	2.8	S
Boron trifluoride dihydrate	13319-75-01	Inorganic acid	1A	(3) 1A	2.4	4.2	4.4	10.1	L
Phenol	108-95-2	Phenol	1A	(3) 1A	29.8	21.8	22.6	13.5	S
Dichloroacetyl chloride	79-36-7	Electrophile	1A	(3) 1A	5.6	6.3	1.3	1.4	L
Combination of sub-categories 1B-and-1C *In Vivo* Corrosives									
Glyoxylic acid monohydrate	563-96-2	Organic acid	1B-and-1C	(3) 1B-and-1C	110.4	22.5	90.4	3.1	S
Lactic acid	598-82-3	Organic acid	1B-and-1C	(3) 1B-and-1C	80.2	9.4	90	3.5	L
Ethanolamine	141-43-5	Organic base	1B	(3) 1B-and-1C	66.2	40.3	69.7	9.3	Viscous
Hydrochloric acid (14.4%)	7647-01-0	Inorganic acid	1B-and-1C	(3) 1B-and-1C	69.3	5.7	80.8	9	L

***In Vivo* Non Corrosives**									
Phenethyl bromide	103-63-9	Electrophile	NC	(3) NC	141	117.2	112.5	71.2	N
4-Amino-1,2,4-triazole	584-13-4	Organic base	NC	(3) NC	116.8	120.6	105.7	88.2	N
4-(methylthio)-benzaldehyde	3446-89-7	Electrophile	NC	(3) NC	136.7	150.4	85.4	81.6	N
Lauric acid	143-07-7	Organic acid	NC	(3) NC	102	117.4	90.7	64.4	N

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; UN GHS = United Nations Globally Harmonized System (1); VRM = Validated Reference Method, EpiSkin™=VRM1, EpiDerm™= VRM2; NC = Not Corrosive

[1]The proficiency substances, sorted first by corrosives versus non-corrosives, then by corrosive sub-category and then by chemical class, were selected from the substances used in the ECVAM validation studies EpiSkin™ and EpiDerm™ (8) (9) (10) and from post-validation studies based on data provided by EpiSkin™ (22), EpiDerm™, SkinEthic™ and epiCS® developers (23). Unless otherwise indicated, the substances were tested at the purity level obtained when purchased from a commercial source (8) (10). The selection includes, to the extent possible, substances that: (i) are representative of the range of corrosivity responses (e.g. non-corrosives; weak to strong corrosives) that the VRMs are capable of measuring or predicting; (ii) are representative of the chemical classes used in the validation studies; (iii) have chemical structures that are well-defined; (iv) induce reproducible results in the VRM; (v) induce definitive results in the in vivo reference test method; (vi) are commercially available; and (vii) are not associated with prohibitive disposal costs.

[2]Chemical class assigned by Barratt et al. (8).

[3]The corresponding UN Packing groups are I, II and III, respectively, for the UN GHS 1A, 1B and 1C.

[4]The in vitro predictions reported in this table were obtained with all five test methods covered in TG 431; for phenol though the LabCyte EPI-MODEL24 had slightly discordant results across runs, i.e. 1A-1BC-1BC; other methods achieved these classifications in validation or post-validation testing performed by the test method developers.

[5]The viability values obtained in the ECVAM Skin Corrosion Validation Studies were not corrected for direct MTT reduction (killed controls were not performed in the validation studies). However, the post-validation data generated by the test method developers that are presented in this table were acquired with adapted controls (23).

14. As part of the proficiency exercise, it is recommended that the user verifies the barrier properties of the tissues after receipt as specified by the RhE model manufacturer. This is particularly important if tissues are shipped over long distance/time periods. Once a test method has been successfully established and proficiency in its use has been demonstrated, such verification will not be necessary on a routine basis. However, when using a test method routinely, it is recommended to continue to assess the barrier properties in regular intervals.

PROCEDURE

15. The following is a generic description of the components and procedures of the RhE test methods for skin corrosion assessment covered by this Test Guideline. The RhE models endorsed as scientifically valid for use within this Test Guideline, i.e. the EpiSkin™ (SM), EpiDerm™ (EPI-200), SkinEthic™ RHE, epiCS® and LabCyte EPI-MODEL24 (16) (17) (19) (28) (29) (30) (31) (32) (33) (40) (41), can be obtained from commercial sources. Standard Operating Procedures (SOPs) for these five RhE models are available (34) (35) (36) (37) (42), and their main test method components are summarised in Annex 2. It is recommended that the relevant SOP be consulted when implementing and using one of these methods in the laboratory. Testing with the five RhE test methods covered by this Test Guideline should comply with the following:

RHE TEST METHOD COMPONENTS

General conditions

16. Non-transformed human keratinocytes should be used to reconstruct the epithelium. Multiple layers of viable epithelial cells (basal layer, stratum spinosum, stratum granulosum) should be present under a functional stratum corneum. The stratum corneum should be multi-layered containing the essential lipid profile to produce a functional barrier with robustness to resist rapid penetration of cytotoxic benchmark chemicals, e.g. sodium dodecyl sulphate (SDS) or Triton X-100. The barrier function should be demonstrated and may be assessed either by determination of the concentration at which a benchmark chemical reduces the viability of the tissues by 50% (IC50) after a fixed exposure time, or by determination of the exposure time required to reduce cell viability by 50% (ET50) upon application of the benchmark chemical at a specified, fixed concentration (see paragraph 18). The containment properties of the RhE model should prevent the passage of material around the stratum corneum to the viable tissue, which would lead to poor modelling of skin exposure. The RhE model should be free of contamination by bacteria, viruses, mycoplasma, or fungi.

Functional conditions

Viability

17. The assay used for quantifying tissue viability is the MTT-assay (27). The viable cells of the RhE tissue construct reduce the vital dye MTT into a blue MTT formazan precipitate, which is then extracted from the tissue using isopropanol (or a similar solvent). The OD of the extraction solvent alone should be sufficiently small, i.e., OD < 0.1. The extracted MTT formazan may be quantified using either a standard absorbance (OD) measurement or an HPLC/UPLC-spectrophotometry procedure (38). The RhE model users should ensure that each batch of the RhE model used meets defined criteria for the negative control. An acceptability range (upper and lower limit) for the negative control OD values should be established by the RhE model developer/supplier. Acceptability ranges for the negative control OD values for the five validated RhE test methods included in this Test Guideline are given in Table 2. An HPLC/UPLC-Spectrophotometry user should use the negative control OD ranges provided in Table 2 as the acceptance criterion for the negative control. It should be documented that the tissues treated with negative control are stable in culture (provide similar OD measurements) for the duration of the exposure period.

Table 2. Acceptability ranges for negative control OD values to control batch quality

	Lower acceptance limit	Upper acceptance limit
EpiSkin™ (SM)	≥ 0.6	≤ 1.5
EpiDerm™ SCT (EPI-200)	≥ 0.8	≤ 2.8
SkinEthic™ RHE	≥ 0.8	≤ 3.0
epiCS	≥ 0.8	≤ 2.8
LabCyte EPI-MODEL24 SCT	≥ 0.7	≤ 2.5

Barrier function

18. The stratum corneum and its lipid composition should be sufficient to resist the rapid penetration of certain cytotoxic benchmark chemicals (e.g. SDS or Triton X-100), as estimated by IC50 or ET50 (Table 3). The barrier function of each batch of the RhE model used should be demonstrated by the RhE model developer/vendor upon supply of the tissues to the end user (see paragraph 21).

Morphology

19. Histological examination of the RhE model should be performed demonstrating multi-layered human epidermis-like structure containing stratum basale, stratum spinosum, stratum granulosum and stratum corneum and exhibits lipid profile similar to lipid profile of human epidermis. Histological examination of each batch of the RhE model used demonstrating appropriate morphology of the tissues should be provided by the RhE model developer/vendor upon supply of the tissues to the end user (see paragraph 21).

Reproducibility

20. Test method users should demonstrate reproducibility of the test methods over time with the positive and negative controls. Furthermore, the test method should only be used if the RhE model developer/supplier provides data demonstrating reproducibility over time with corrosive and non-corrosive chemicals from e.g. the list of Proficiency Substances (Table 1). In case of the use of a test method for sub-categorisation, the reproducibility with respect to sub-categorisation should also be demonstrated.

Quality control (QC)

21. The RhE model should only be used if the developer/supplier demonstrates that each batch of the RhE model used meets defined production release criteria, among which those for viability (paragraph 17), barrier function (paragraph 18) and morphology (paragraph 19) are the most relevant. These data are provided to the test method users, so that they are able to include this information in the test report. Only results produced with QC accepted tissue batches can be accepted for reliable prediction of corrosive classification. An acceptability range (upper and lower limit) for the IC50 or the ET50 is established by the RhE model developer/supplier. The acceptability ranges for the five validated test methods are given in Table 3.

Table 3. **QC batch release criterion**

	Lower acceptance limit	Upper acceptance limit
EpiSkin™ (SM) (18 hours treatment with SDS)(33)	IC_{50} = 1.0 mg/mL	IC_{50} = 3.0 mg/mL
EpiDerm™SCT (EPI-200) (1% Triton X-100)(34)	ET_{50} = 4.0 hours	ET_{50} = 8.7 hours
SkinEthic™ RHE (1% Triton X-100)(35)	ET_{50} = 4.0 hours	ET_{50} = 10.0 hours
epiCS (1% Triton X-100)(36)	ET_{50} = 2.0 hours	ET_{50} = 7.0 hours
LabCyte EPI-MODEL24 SCT (18 hours treatment with SDS) (42)	IC_{50} = 1.4 mg/mL	IC_{50} = 4.0 mg/mL

Application of the Test Chemical and Control Substances

22. At least two tissue replicates should be used for each test chemical and controls for each exposure time. For liquid as well as solid chemicals, sufficient amount of test chemical should be applied to uniformly cover the epidermis surface while avoiding an infinite dose, i.e. a minimum of 70 µL/cm2 or 30 mg/cm2 should be used. Depending on the methods, the epidermis surface should be moistened with deionized or distilled water before application of solid chemicals, to improve contact between the test chemical and the epidermis surface (34) (35) (36) (37) (42). Whenever possible, solids should be tested as a fine powder. The application method should be appropriate for the test chemical (see e.g. references (34-37). At the end of the exposure period, the test chemical should be carefully washed from the epidermis with an aqueous buffer, or 0.9% NaCl. Depending on which of the five validated RhE test methods is used, two or three exposure periods are used per test chemical (for all five valid RhE models: 3 min and 1 hour; for EpiSkin™ an additional exposure time of 4 hours). Depending on the RhE test method used and the exposure period assessed, the incubation temperature during exposure may vary between room temperature and 37ºC.

23. Concurrent negative and positive controls (PC) should be used in each run to demonstrate that viability (with negative controls), barrier function and resulting tissue sensitivity (with the PC) of the tissues are within a defined historical acceptance range. The suggested PC chemicals are glacial acetic acid or 8N KOH depending upon the RhE model used (see Annex 2 and relevant SOP for details). It should be noted that 8N KOH is a direct MTT reducer that might require adapted controls as described in paragraphs 25 and 26. The suggested negative controls are 0.9% (w/v) NaCl or water.

Cell Viability Measurements

24. The MTT assay, which is a quantitative assay, should be used to measure cell viability under this Test Guideline (27). The tissue sample is placed in MTT solution of appropriate concentration (0.3, 0.5 or 1 mg/mL, see Annex 2 and relevant SOP for details) for 3 hours. The precipitated blue formazan product is then extracted from the tissue using a solvent (e.g. isopropanol, acidic isopropanol), and the concentration of formazan is

measured by determining the OD at 570 nm using a filter band pass of maximum ± 30 nm, or by an HPLC/UPLC spectrophotometry procedure (see paragraphs 30 and 31) (38).

25. Test chemicals may interfere with the MTT assay, either by direct reduction of the MTT into blue formazan, and/or by colour interference if the test chemical absorbs, naturally or due to treatment procedures, in the same OD range of formazan (570 ± 30 nm, mainly blue and purple chemicals). Additional controls should be used to detect and correct for a potential interference from these test chemicals such as the non-specific MTT reduction (NSMTT) control and the non-specific colour (NSC) control (see paragraphs 26 to 30). This is especially important when a specific test chemical is not completely removed from the tissue by rinsing or when it penetrates the epidermis, and is therefore present in the tissues when the MTT viability test is performed. Detailed description of how to correct direct MTT reduction and interferences by colouring agents is available in the SOPs for the test methods (34) (35) (36) (37) (42).

26. To identify direct MTT reducers, each test chemical should be added to freshly prepared MTT medium (34) (35) (36) (37) (42). If the MTT mixture containing the test chemical turns blue/purple, the test chemical is presumed to directly reduce the MTT, and further functional check on non-viable epidermis should be performed, independently of using the standard absorbance (OD) measurement or an HPLC/UPLC-spectrophotometry procedure. This additional functional check employs killed tissues that possess only residual metabolic activity but absorb the test chemical in similar amount as viable tissues. Each MTT reducing chemical is applied on at least two killed tissue replicates per exposure time, which undergo the whole skin corrosion test. The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the MTT reducer minus the percent non-specific MTT reduction obtained with the killed tissues exposed to the same MTT reducer, calculated relative to the negative control run concurrently to the test being corrected (%NSMTT).

27. To identify potential interference by coloured test chemicals or test chemicals that become coloured when in contact with water or isopropanol and decide on the need for additional controls, spectral analysis of the test chemical in water (environment during exposure) and/or isopropanol (extracting solution) should be performed. If the test chemical in water and/or isopropanol absorbs light in the range of 570 ± 30 nm, further colorant controls should be performed or, alternatively, an HPLC/UPLC-spectrophotometry procedure should be used in which case these controls are not required (see paragraphs 30 and 31). When performing the standard absorbance (OD) measurement, each interfering coloured test chemical is applied on at least two viable tissue replicates per exposure time, which undergo the entire skin corrosion test but are incubated with medium instead of MTT solution during the MTT incubation step to generate a non-specific colour (NSCliving) control. The NSCliving control needs to be performed concurrently per exposure time per coloured test chemical (in each run) due to the inherent biological variability of living tissues. The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the interfering test chemical and incubated with MTT solution minus the percent non-specific colour obtained with living tissues exposed to the interfering test chemical and incubated with medium without MTT, run concurrently to the test being corrected (%NSCliving).

28. Test chemicals that are identified as producing both direct MTT reduction (see paragraph 26) and colour interference (see paragraph 27) will also require a third set of controls, apart from the NSMTT and NSCliving controls described in the previous

paragraphs, when performing the standard absorbance (OD) measurement. This is usually the case with darkly coloured test chemicals interfering with the MTT assay (e.g., blue, purple, black) because their intrinsic colour impedes the assessment of their capacity to directly reduce MTT as described in paragraph 26. These test chemicals may bind to both living and killed tissues and therefore the NSMTT control may not only correct for potential direct MTT reduction by the test chemical, but also for colour interference arising from the binding of the test chemical to killed tissues. This could lead to a double correction for colour interference since the NSCliving control already corrects for colour interference arising from the binding of the test chemical to living tissues. To avoid a possible double correction for colour interference, a third control for non-specific colour in killed tissues (NSCkilled) needs to be performed. In this additional control, the test chemical is applied on at least two killed tissue replicates per exposure time, which undergo the entire testing procedure but are incubated with medium instead of MTT solution during the MTT incubation step. A single NSCkilled control is sufficient per test chemical regardless of the number of independent tests/runs performed, but should be performed concurrently to the NSMTT control and, where possible, with the same tissue batch. The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the test chemical minus %NSMTT minus %NSCliving plus the percent non-specific colour obtained with killed tissues exposed to the interfering test chemical and incubated with medium without MTT, calculated relative to the negative control run concurrently to the test being corrected (%NSCkilled).

29. It is important to note that non-specific MTT reduction and non-specific colour interferences may increase the readouts of the tissue extract above the linearity range of the spectrophotometer. On this basis, each laboratory should determine the linearity range of their spectrophotometer with MTT formazan (CAS # 57360-69-7) from a commercial source before initiating the testing of test chemicals for regulatory purposes. In particular, the standard absorbance (OD) measurement using a spectrophotometer is appropriate to assess direct MTT-reducers and colour interfering test chemicals when the ODs of the tissue extracts obtained with the test chemical without any correction for direct MTT reduction and/or colour interference are within the linear range of the spectrophotometer or when the uncorrected percent viability obtained with the test chemical already defined it as a corrosive (see paragraphs 35 and 36). Nevertheless, results for test chemicals producing %NSMTT and/or %NSCliving ≥ 50% of the negative control should be taken with caution.

30. For coloured test chemicals which are not compatible with the standard absorbance (OD) measurement due to too strong interference with the MTT assay, the alternative HPLC/UPLC-spectrophotometry procedure to measure MTT formazan may be employed (see paragraph 31) (37). The HPLC/UPLC-spectrophotometry system allows for the separation of the MTT formazan from the test chemical before its quantification (38). For this reason, NSCliving or NSCkilled controls are never required when using HPLC/UPLC-spectrophotometry, independently of the chemical being tested. NSMTT controls should nevertheless be used if the test chemical is suspected to directly reduce MTT or has a colour that impedes the assessment of the capacity to directly reduce MTT (as described in paragraph 26). When using HPLC/UPLC-spectrophotometry to measure MTT formazan, the percent tissue viability is calculated as percent MTT formazan peak area obtained with living tissues exposed to the test chemical relative to the MTT formazan peak obtained with the concurrent negative control. For test chemicals able to directly reduce MTT, true tissue viability is calculated as the percent tissue viability obtained with living tissues exposed to the test chemical minus %NSMTT. Finally, it should be noted that direct MTT-reducers

that may also be colour interfering, which are retained in the tissues after treatment and reduce MTT so strongly that they lead to ODs (using standard OD measurement) or peak areas (using UPLC/HPLC-spectrophotometry) of the tested tissue extracts that fall outside of the linearity range of the spectrophotometer cannot be assessed, although these are expected to occur in only very rare situations.

31. HPLC/UPLC-spectrophotometry may be used also with all types of test chemicals (coloured, non-coloured, MTT-reducers and non-MTT reducers) for measurement of MTT formazan (38). Due to the diversity of HPLC/UPLC-spectrophotometry systems, qualification of the HPLC/UPLC-spectrophotometry system should be demonstrated before its use to quantify MTT formazan from tissue extracts by meeting the acceptance criteria for a set of standard qualification parameters based on those described in the U.S. Food and Drug Administration guidance for industry on bio-analytical method validation (38) (39). These key parameters and their acceptance criteria are shown in Annex 4. Once the acceptance criteria defined in Annex 4 have been met, the HPLC/UPLC-spectrophotometry system is considered qualified and ready to measure MTT formazan under the experimental conditions described in this Test Guideline.

Acceptance Criteria

32. For each test method using valid RhE models, tissues treated with the negative control should exhibit OD reflecting the quality of the tissues as described in table 2 and should not be below historically established boundaries. Tissues treated with the PC, i.e. glacial acetic acid or 8N KOH, should reflect the ability of the tissues to respond to a corrosive chemical under the conditions of the test method (see Annex 2 and relevant SOP for details). The variability between tissue replicates of test chemical and/or control substances should fall within the accepted limits for each valid RhE model requirements (see Annex 2 and relevant SOP for details) (e.g. the difference of viability between the two tissue replicates should not exceed 30%). If either the negative control or PC included in a run fall out of the accepted ranges, the run is considered as not qualified and should be repeated. If the variability of test chemicals falls outside of the defined range, its testing should be repeated.

Interpretation of Results and Prediction Model

33. The OD values obtained for each test chemical should be used to calculate percentage of viability relative to the negative control, which is set at 100%. In case HPLC/UPLC-spectrophotometry is used, the percent tissue viability is calculated as percent MTT formazan peak area obtained with living tissues exposed to the test chemical relative to the MTT formazan peak obtained with the concurrent negative control. The cut-off percentage cell viability values distinguishing corrosive from non-corrosive test chemical (or discriminating between different corrosive sub-categories) are defined below in paragraphs 35 and 36 for each of the test methods covered by this Test Guideline and should be used for interpreting the results.

34. A single testing run composed of at least two tissue replicates should be sufficient for a test chemical when the resulting classification is unequivocal. However, in cases of borderline results, such as non-concordant replicate measurements, a second run may be considered, as well as a third one in case of discordant results between the first two runs.

35. The prediction model for the EpiSkin™ skin corrosion test method (9) (34) (22), associated with the UN GHS (1) classification system, is shown in Table 4:

Table 4. EpiSkin™ prediction model

Viability measured after exposure time points (t='3,' 60 and 240 minutes)	Prediction to consider
< 35% after 3 min exposure	**Corrosive:** Optional Sub-category 1A *
≥ 35% after 3 min exposure **AND** < 35% after 60 min exposure **OR** ≥ 35% after 60 min exposure **AND** < 35% after 240 min exposure	**Corrosive:** A combination of optional Sub-categories 1B-and-1C
≥ 35% after 240 min exposure	**Non-corrosive**

*) According to the data generated in view of assessing the usefulness of the RhE test methods for supporting sub-categorisation, it was shown that around 22 % of the Sub-category 1A results of the EpiSkin™ test method may actually constitute Sub-category 1B or Sub-category 1C substances/mixtures (i.e. over classifications) (see Annex 3).

36. The prediction models for the EpiDerm™ SCT (10) (23) (35), the SkinEthic™ RHE (17) (18) (23) (36), the epiCS® (16) (23) (37) and LabCyte EPI-MODEL24 (41) (42) skin corrosion test methods, associated with the UN GHS (1) classification system, are shown in Table 5:

Table 5. EpiDerm™ SCT, SkinEthic™ RHE epiCS® and LabCyte EPI-MODEL24 SCT

Viability measured after exposure time points (t=3 and 60 minutes)	Prediction to be considered
STEP 1 for EpiDerm™ SCT, SkinEthic™ RHE, epiCS® and LabCyte EPI-MODEL24 SCT	
< 50% after 3 min exposure	Corrosive
≥ 50% after 3 min exposure AND < 15% after 60 min exposure	Corrosive
≥ 50% after 3 min exposure AND ≥ 15% after 60 min exposure	Non-corrosive
STEP 2 for EpiDerm™ SCT - for substances/mixtures identified as Corrosive in step 1	
< 25% after 3 min exposure	Optional Sub-category 1A *
≥ 25% after 3 min exposure	A combination of optional Sub-categories 1B-and-1C
STEP 2 for SkinEthic™ RHE - for substances/mixtures identified as Corrosive in step 1	
< 18% after 3 min exposure	Optional Sub-category 1A *
≥ 18% after 3 min exposure	A combination of optional Sub-categories 1B-and-1C
STEP 2 for epiCS® - for substances/mixtures identified as Corrosive in step 1	
< 15% after 3 min exposure	Optional Sub-category 1A *
≥ 15% after 3 min exposure	A combination of optional Sub-categories 1B-and-1C
STEP 2 for LabCyte EPI-MODEL24 SCT - for substances/mixtures identified as Corrosive in step 1	
< 15% after 3 min exposure	Optional Sub-category 1A *
≥ 15% after 3 min exposure	A combination of optional Sub-categories 1B-and-1C

* According to the data generated in view of assessing the usefulness of the RhE test methods for supporting sub-categorisation, it was shown that around 29%, 31%, 33% and 30% of the Sub-category 1A results of the EpiDerm™ SCT, SkinEthic™ RHE epiCS® and LabCyte EPI-MODEL24 SCT, respectively, may actually constitute Sub-category 1B or Sub-category 1C substances/mixtures (i.e. over-classifications) (see Annex 3).

DATA AND REPORTING

Data

37. For each test, data from individual tissue replicates (e.g. OD values and calculated percentage cell viability for each test chemical, including classification) should be reported in tabular form, including data from repeat experiments as appropriate. In addition, means and ranges of viability and CVs between tissue replicates for each test should be reported. Observed interactions with MTT reagent by direct MTT reducers or coloured test chemicals should be reported for each tested chemical.

Test Report

38. The test report should include the following information:

Test Chemical and Control Substances:

- Mono-constituent substance: chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc;
- Multi-constituent substance, UVCB and mixture: characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents;
- Physical appearance, water solubility, and any additional relevant physicochemical properties;
- Source, lot number if available;
- Treatment of the test chemical/control substance prior to testing, if applicable (e.g. warming, grinding);
- Stability of the test chemical, limit date for use, or date for re-analysis if known;
- Storage conditions.

RhE model and protocol used and rationale for it (if applicable)

Test Conditions:

- RhE model used (including batch number);
- Calibration information for measuring device (e.g. spectrophotometer), wavelength and band
- pass (if applicable) used for quantifying MTT formazan, and linearity range of measuring device;
- Description of the method used to quantify MTT formazan;
- Description of the qualification of the HPLC/UPLC-spectrophotometry system, if applicable;
- Complete supporting information for the specific RhE model used including its performance. This should include, but is not limited to:
    - i) Viability;
    - ii) Barrier function;
    - iii) Morphology;
    - iv) Quality controls (QC) of the model;
- Reference to historical data of the model. This should include, but is not limited to acceptability of the QC data with reference to historical batch data;
- Demonstration of proficiency in performing the test method before routine use by testing of the proficiency substances.

Test Procedure:

- Details of the test procedure used (including washing procedures used after exposure period);
- Doses of test chemical and control substances used;
- Duration of exposure period(s) and temperature(s) of exposure;
- Indication of controls used for direct MTT-reducers and/or colouring test chemicals, if applicable;
- Number of tissue replicates used per test chemical and controls (PC, negative control, and NSMTT, NSCliving and NSCkilled, if applicable), per exposure time;
- Description of decision criteria/prediction model applied based on the RhE model used;
- Description of any modifications of the test procedure (including washing procedures).
- Run and Test Acceptance Criteria:
- Positive and negative control mean values and acceptance ranges based on historical data;
- Acceptable variability between tissue replicates for positive and negative controls;
- Acceptable variability between tissue replicates for test chemical.

Results:

- Tabulation of data for individual test chemicals and controls, for each exposure period, each run and each replicate measurement including OD or MTT formazan peak area, percent tissue viability, mean percent tissue viability, differences between replicates, SDs and/or CVs if applicable;
- If applicable, results of controls used for direct MTT-reducers and/or colouring test chemicals including OD or MTT formazan peak area, %NSMTT, %NSCliving, %NSCkilled, differences between tissue replicates, SDs and/or CVs (if applicable), and final correct percent tissue viability;
- Results obtained with the test chemical(s) and control substances in relation to the defined run and test acceptance criteria;
- Description of other effects observed;
- The derived classification with reference to the prediction model/decision criteria used.

Discussion of the results:

Conclusions:

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ANNEX 1- DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with "concordance" to mean the proportion of correct outcomes of a test method (25).

Cell viability: Parameter measuring total activity of a cell population e.g. as ability of cellular mitochondrial dehydrogenases to reduce the vital dye MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, Thiazolyl blue), which depending on the endpoint measured and the test design used, correlates with the total number and/or vitality of living cells.

Chemical: means a substance or a mixture.

Concordance: This is a measure of test method performance for test methods that give a categorical result, and is one aspect of relevance. The term is sometimes used interchangeably with accuracy, and is defined as the proportion of all chemicals tested that are correctly classified as positive or negative. Concordance is highly dependent on the prevalence of positives in the types of test chemical being examined (25).

ET50: Can be estimated by determination of the exposure time required to reduce cell viability by 50% upon application of the benchmark chemical at a specified, fixed concentration, see also IC50.

GHS (Globally Harmonized System of Classification and Labelling of Chemicals): A system proposing the classification of chemicals (substances and mixtures) according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (1).

HPLC: High Performance Liquid Chromatography.

IATA: Integrated Approach on Testing and Assessment.

IC50: Can be estimated by determination of the concentration at which a benchmark chemical reduces the viability of the tissues by 50% (IC50) after a fixed exposure time, see also ET50.

ET50. Infinite dose: Amount of test chemical applied to the epidermis exceeding the amount required to completely and uniformly cover the epidermis surface.

Mixture: means a mixture or solution composed of two or more substances in which they do not react.

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide.

Multi-constituent substance: A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration ≥ 10% (w/w) and <

80% (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

NC: Non corrosive.

NSCkilled control: Non-Specific Colour control in killed tissues.

NSCliving control : Non-Specific Colour control in living tissues.

NSMTT: Non-Specific MTT reduction.

OD: Optical Density

PC: Positive Control, a replicate containing all components of a test system and treated with a substance known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Performance standards (PS): Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are; (i) essential test method components; (ii) a minimum list of Reference Chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (iii) the similar levels of reliability and accuracy, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of Reference Chemicals (25).

Relevance: Description of relationship of the test method to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test method correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (25).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility (25).

Run: A run consists of one or more test chemicals tested concurrently with a negative control and with a PC.

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (25).

Skin corrosion in vivo: The production of irreversible damage of the skin; namely, visible necrosis through the epidermis and into the dermis, following the application of a test chemical for up to four hours. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, and, by the end of observation at 14 days, by discoloration due to blanching of the skin, complete areas of alopecia, and scars. Histopathology should be considered to evaluate questionable lesions.

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (25).

Substance: means chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the

product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.

Test chemical: means what is being tested.

UPLC: Ultra-High Performance Liquid Chromatography.

UVCB: substances of unknown or variable composition, complex reaction products or biological materials.

ANNEX 2 - MAIN TEST METHOD COMPONENTS OF THE RhE TEST METHODS VALIDATED FOR SKIN CORROSION TESTING

Nr.	1	2	3	4	5
Test Method Component	**EpiSkin™**	**EpiDerm™ SCT**	**SkinEthic™ RHE**	**epiCS®**	**LabCyte EPI-MODEL24 SCT**
Model surface	0.38 cm^2	0.63 cm^2	0.5 cm^2	0.6 cm^2	0.3 cm^2
Number of tissue replicates	At least 2 per exposure time	2-3 per exposure time	At least 2 per exposure time	At least 2 per exposure time	At least 2 per exposure time
Treatment doses and application	Liquids and viscous: 50 ± 3 µL (131.6 µL/cm^2) Solids: 20± 2 mg (52.6 mg/cm^2) +100 µL±5µL NaCl solution (9 g/L) Waxy/sticky: 50 ± 2 mg (131.6 mg/cm^2) with a nylon mesh	Liquids: 50 µL (79.4 µL/cm^2) with or without a nylon mesh *Pre-test compatibility of test chemical with nylon mesh* Semi solids: 50 µL (79.4 µL/cm^2) Solids: 25 µL H_2O (or necessary) + 25 mg (39.7 mg/cm^2) Waxes: flat "disc like" piece of ca. 8 mm diameter placed atop the tissue wetted with 15µL H_2O.	Liquids and viscous:40 ± 3 µL (80µL/cm^2) using nylonmesh *Pre-test compatibility of test chemical with nylon mesh* Solids: 20 µL ± 2µl H_2O + 20± 3 mg (40 mg/cm2) Waxy/sticky: 20 ± 3 mg (40 mg/cm^2) with a nylon mesh	Liquids and viscous:50 µL (83.3µL/cm^2) using nylonmesh *Pre-test compatibility of test chemical with nylon mesh* Semi solids: 50 µL (83.3 µL/cm^2) Solids: 25 mg (41.7 mg/cm^2) + 25 µL H_2O (or more if necessary) Waxy/sticky: flat "cookie like" piece of ca. 8 mm diameter placed atop the tissue wetted with 15µL H_2O	Liquids and viscous:50 µL (166.7µL/cm^2) Solids: 50± 2 mg (166.7 mg/cm^2) + 50 µL H_2O Waxy: Use a positive displacement pipette and tip as liquid and viscous substance.

Nr.	1	2	3	4	5
Test Method Component	**EpiSkin™**	**EpiDerm™ SCT**	**SkinEthic™ RHE**	**epiCS®**	**LabCyte EPI-MODEL24 SCT**
Pre-check for direct MTT reduction	50 µL (liquid) or 20 mg (solid) + 2 mL MTT 0.3 mg/mL solution for 180±5 min at 37°C, 5% CO_2, 95% RH ➔ if solution turns blue/purple, water-killed adapted controls should be performed	50 µL (liquid) or 25 mg (solid) + 1 mL MTT 1 mg/mL solution for 60 min at 37°C, 5% CO_2, 95% RH ➔ if solution turns blue/purple, freeze-killed adapted controls should be performed	40 µL (liquid) or 20 mg (solid) + 1 mL MTT 1 mg/mL solution for 180±15 min at 37°C, 5% CO_2, 95% RH ➔ if solution turns blue/purple, freeze-killed adapted controls should be performed	50 µL (liquid) or 25 mg (solid) + 1 mL MTT 1 mg/mL solution for 60 min at 37°C, 5% CO_2, 95% RH ➔ if solution turns blue/purple, freeze-killed adapted controls should be performed	50 µL (liquid) or 50 mg (solid) + 500 µL MTT 0.5 mg/mL solution for 60 min at 37°C, 5% CO_2, 95% RH ➔ if solution turns blue/purple, freeze-killed adapted controls should be performed
Pre-check for colour interference	10 µL (liquid) or 10 mg (solid) + 90µL H2O mixed for 15 min at RT ➔ if solution becomes coloured, living adapted controls should be performed	50 µL (liquid) or 25 mg (solid) + 300 µL H2O mixed for 60 min at 37oC, 5% CO2, 95% RH ➔ if solution becomes coloured, living adapted controls should be performed	40 µL (liquid) or 20 mg (solid) + 300 µL H2O mixed for 60 min at RT ➔ if solution becomes coloured, living adapted controls should be performed	50 µL (liquid) or 25 mg (solid) + 300 µL H2O mixed for 60 min at 37oC, 5% CO2, 95% RH ➔ if solution becomes coloured, living adapted controls should be performed	50 µL (liquid) or 50 mg (solid) + 500 µL H2O mixed for 60 min at 37oC, 5% CO2, 95% RH ➔ if solution becomes coloured, living adapted controls should be performed
Exposure time and temperature	3 min, 60 min (±5 min) and 240 min (±10 min) In ventilated cabinet Room Temperature (RT, 18-28oC)	3 min at RT, and 60 min at 37oC, 5% CO2, 95% RH	3 min at RT, and 60 min at 37oC, 5% CO2, 95% RH	3 min at RT, and 60 min at 37oC, 5% CO2, 95% RH	3 min at RT, and 60 min at 37oC, 5% CO2, 95% RH
Rinsing	25 mL 1x PBS (2 mL/throwing)	20 times with a constant soft stream of 1x PBS	20 times with a constant soft stream of 1x PBS	20 times with a constant soft stream of 1x PBS	10 times or more with a constant strong stream of 1x PBS
Negative control	50 µL NaCl solution (9 g/L) Tested with every exposure time	50 µL H2O Tested with every exposure time	40 µL H2O Tested with every exposure time	50 µL H2O Tested with every exposure time	50 µL H2O Tested with every exposure time
Positive control	50 µL Glacial acetic acid Tested only for 4 hours	50 µL 8N KOH Tested with every exposure time	40 µL 8N KOH Tested only for 1 hour	50 µL 8N KOH Tested with every exposure time	50 µL 8N KOH Tested only for 1 hour
MTT solution	2 mL 0.3 mg/mL	300 µL 1 mg/mL	300 µL 1 mg/mL	300 µL 1 mg/mL	500 µL 0.5 mg/mL
MTT incubation	180 min (±15 min) at 37oC, 5%	180 min at 37oC, 5% CO2, 95%	180 min (±15 min) at 37oC, 5%	180 min at 37oC, 5% CO2, 95%	180 min (±5 min) at 37oC, 5%

Nr.	1	2	3	4	5
Test Method Component	**EpiSkin™**	**EpiDerm™ SCT**	**SkinEthic™ RHE**	**epiCS®**	**LabCyte EPI-MODEL24 SCT**
time and temperature	CO2, 95% RH	RH	CO2, 95% RH	RH	CO2, 95% RH
Test Method Component	EpiSkin™ EIT	EpiDerm™ SCT	SkinEthic™ RHE EIT	epiCS®	LabCyte EPI-MODEL24 SCT
Extraction solvent	500 µL acidified isopropanol (0.04 N HCl in isopropanol) (isolated tissue fully immersed)	2 mL isopropanol (extraction from top and bottom of insert)	1.5 mL isopropanol (extraction from top and bottom of insert)	2 mL isopropanol (extraction from top and bottom of insert)	300 µL isopropanol (isolated tissue fully immersed)
Extraction time And temperature	Overnight at RT, protected from light	Overnight without shaking at RT or for 120 min with shaking (~120 rpm) at RT	Overnight without shaking at RT or for 120 min with shaking (~120 rpm) at RT	Overnight without shaking at RT or for 120 min with shaking (~120 rpm) at RT	Overnight at RT, protected from light
OD reading	570 nm (545 - 595 nm) without reference filter	570 nm (or 540 nm) without reference filter	570 nm (540 - 600 nm) without reference filter	540 - 570 nm without reference filter	570 nm with reference filter 650 nm
Tissue Quality Control	18 hours treatment with SDS 1.0mg/mL ≤ IC_{50} ≤ 3.0mg/mL	Treatment with 1% Triton X-100 4.08 hours ≤ ET_{50} ≤ 8.7 hours	Treatment with 1% Triton X-100 4.0 hours ≤ ET_{50} ≤ 10.0 hours	Treatment with 1% Triton X-100 2.0 hours ≤ ET_{50} ≤ 7.0 hours	18 hours treatment with SDS 1.4mg/mL ≤ IC_{50} ≤ 4.0 mg/mL
Acceptability Criteria	1. Mean OD of the tissue replicates treated with the negative control (NaCl) should be ≥ 0.6 and ≤ 1.5 for every exposure time 2. Mean viability of the tissue replicates exposed for 4 hours with the positive control (glacial acetic acid), expressed as % of the negative control, should be ≤ 20% 3. In the range 20-100% viability and for ODs ≥ 0.3, difference of viability between the two tissue replicates should not	1. Mean OD of the tissue replicates treated with the negative control (H_2O) should be ≥ 0.8 and ≤ 2.8 for every exposure time 2. Mean viability of the tissue replicates exposed for 1 hour with the positive control (8N KOH), expressed as % of the negative control, should be ≤ 15% 3. In the range 20 - 100% viability, the Coefficient of Variation (CV) between tissue replicates should be ≤ 30%	1. Mean OD of the tissue replicates treated with the negative control (H_2O) should be ≥ 0.8 and ≤ 3.0 for every exposure time 2. Mean viability of the tissue replicates exposed for 1 hour (and 4 hours, if applicable) with the positive control (8N KOH), expressed as % of the negative control, should be ≤ 15% 3. In the range 20-100% viability and for ODs ≥ 0.3, difference of viability between the two	1. Mean OD of the tissue replicates treated with the negative control (H_2O) should be ≥ 0.8 and ≤ 2.8 for every exposure time 2. Mean viability of the tissue replicates exposed for 1 hour with the positive control (8N KOH), expressed as % of the negative control, should be ≤ 15%. 3. In the range 20-100% viability and for ODs ≥ 0.3, difference of viability between the two tissue replicates should not	1. Mean OD of the tissue replicates treated with the negative control (H_2O) should be ≥ 0.7 and ≤ 2.5 for every exposure time 2. Mean viability of the tissue replicates exposed for 1 hour with the positive control (8N KOH), expressed as % of the negative control, should be ≤ 15%. 3. In the range 20-100% viability and for ODs ≥ 0.3, difference of viability between the two tissue replicates should not

Nr.	1	2	3	4	5
Test Method Component	EpiSkin™	EpiDerm™ SCT	SkinEthic™ RHE	epiCS®	LabCyte EPI-MODEL24 SCT
	exceed 30%.		tissue replicates should not exceed 30%.	exceed 30%.	exceed 30%.

ANNEX 3 - PERFORMANCE OF TEST METHODS FOR SUB-CATEGORISATION

The table below provides the performances of the five test methods calculated based on a set of 79 or 80 chemicals tested by the five test developers. Calculations of four test methods (EpiSkin™, EpiDerm™ SCT, SkinEthic™ RHE and epiCS®) were performed by the OECD Secretariat, reviewed and agreed by an expert subgroup (21) (23). Calculation of LabCyte EPI-MODEL24 SCT was performed by the test developer, reviewed and agreed by the validation management group and a peer review panel (41) (43).

STATISTICS ON PREDICTIONS OBTAINED ON THE ENTIRE SET OF CHEMICALS (n= 80 chemicals tested over 2 independent runs for epiCS® or 3 independent runs for EpiDerm™ SCT, EpiSkin™ and SkinEthic™RHE *i.e.* respectively 159* or 240 classifications.
n= 79 chemicals tested over 3 independent runs for LabCyte EPI-MODEL24 SCT, *i.e.* 237 classification.)**
*one chemical was tested once in epiCS® because of no availability (23).
** one chemical was not tested in LabCyte EPI-MODEL24 SCT because of no availability.

	EpiSkin	**EpiDerm**	**SkinEthic**	**epiCS**	**LabCyte EPI-MODEL24**
Overclassifications:					
1B-and-1C overclassified 1A	21.5%	29.0%	31.2%	32.8%	30.0%
NC overclassified 1B-and-1C	20.7%	23.4%	27.0%	28.4%	18.9%
NC overclassified 1A	0.0%	2.7%	0.0%	0.0%	2.7%
Overclassified as Corrosive	20.7%	26.1%	27.0%	28.4%	21.6%
Global overclassification rate (all categories)	17.9%	23.3%	24.5%	25.8%	21.5%
Underclassifications:					
1A underclassified 1B-and-1C	16.7%	16.7%	16.7%	12.5%	13.9%
1A underclassified NC	0.0%	0.0%	0.0%	0.0%	0.0%
1B-and-1C underclassified NC	2.2%	0.0%	7.5%	6.6%	0.0%
Global underclassification rate (all categories)	3.3%	2.5%	5.4%	4.4%	2.1%
Correct Classifications:					
1A correctly classified	83.3%	83.3%	83.3%	87.5%	86.1%
1B-and-/1C correctly classified	76.3%	71.0%	61.3%	60.7%	70.0%
NC correctly classified	79.3%	73.9%	73.0%	71.62%	78.4%
Overall Accuracy	78.8%	74.2%	70.0%	69.8%	76.4%

ANNEX 4 - Key parameters and acceptance criteria for qualification of an HPLC/UPLC-spectrophotometry system for measurement of MTT formazan extracted from RhE tissues

Parameter	Protocol Derived from FDA Guidance (36)(38)	Acceptance Criteria
Selectivity	Analysis of isopropanol, living blank (isopropanol extract from living RhE tissues without any treatment), dead blank (isopropanol extract from killed RhE tissues without any treatment)	$Area_{interference}$ = 20% of $Area_{LLOQ}$[1]
Precision	Quality Controls (i.e., MTT formazan at 1.6 g/mL, 16 g/mL and 160 g/mL) in isopropanol (n=5)	CV = 15% or = 20% for the LLOQ
Accuracy	Quality Controls in isopropanol (n=5)	%Dev = 15% or = 20% for LLOQ
Matrix Effect	Quality Controls in living blank (n=5)	85% = %Matrix Effect= 115%
Carryover	Analysis of isopropanol after an ULOQ[2] standard	$Area_{interference}$ = 20% of $Area_{LLOQ}$
Reproducibility (intra-day)	3 independent calibration curves (based on 6 consecutive 1/3 dilutions of MTT formazan in isopropanol starting at ULOQ, i.e., 200 g/mL); Quality Controls in isopropanol (n=5)	Calibration Curves:%Dev = 15% or = 20% for LLOQ Quality Controls:
Reproducibility (inter-day)	Day 1: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 2: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 3: 1 calibration curve and Quality Controls in isopropanol (n=3)	%Dev= 15% and CV = 15%
Short Term Stability of MTT Formazan in RhE Tissue Extract	Quality Controls in living blank (n='3)' analysed the day of the preparation and after 24 hours of storage at room temperature	%Dev = 15%
Long Term Stability of MTT Formazan in RhE Tissue Extract, if required	Quality Controls in living blank (n='3)' analysed theday of the preparation and after several days of storageat a specified temperature (e.g., 4ºC, -20ºC, -80ºC)	%Dev = 15%

Note:
[1]LLOQ: Lower Limit of Quantification, defined to cover 1-2% tissue viability, i.e., 0.8 µg/mL.
[2]ULOQ: Upper Limit of Quantification, defined to be at least two times higher than the highest expected MTT formazan concentration in isopropanol extracts from negative controls i.e., 200 µg/mL.

IL-2 Luciferase (IL-2 Luc) Assay Report of the Peer Review Panel

on

a JaCVAM co-ordinated study programme addressing the validation status of the IL-2 Luc assay for the prospective identification of immunotoxicic substances on T-cells

Report completed by the Peer review Panel on May XX, 2020

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Executive Summary

The IL-2 Luc assay has been proposed as an in vitro alternative, providing information on in the adverse outcome pathway (AOP) for immunotoxicity, especially on T-Cells. This assay identifies the effects of chemicals on the IL-2 luciferase (Luc) activity.

The peer review panel (PRP) found the Validation Management Team's report presented the necessary information for an independent review.

Consequently, the PRP were able to conclude that the IL-2 Luc assay was well defined, with a clear protocol and criteria for data interpretation. Both within and between laboratory reproducibility information were satisfactory. On the other, the predictive capacity was not satisfactory as a stand alone method. All necessary information including performance standards were detailed.

The PRP should stress this assay is that it is not intended to be used as a sole indicator of immunotoxicity and the reliability of the criteria about the immunotoxicological chemicals is to be discussed further.

Accordingly, the PRP concluded that the IL-2 Luc assay validation has demonstrated that the method would be acceptable as part of an integrated testing strategy for the predictive screening of T-cell targeted immunotoxicity.

Peer Review Panel Composition

Fujio Kayama (chair)	Jichi Medical University, Japan
Henk van Loveren	Maastricht University, Netherland
Haley LaNef Ford	Seattle Genetics, Inc., USA
Barbara Kaplan	Mississippi State University, USA
Xingchao Geng	National Center for Safety Evaluation of Drugs (NCSED), China
Takao Ashikaga (Vice chair)	JaCVAM, Kawasaki, Japan
Sang-Hyun Kim	Kyungpook National University, Korea

Background

Immune dysregulation can have serious adverse health consequences and it could be caused by many types of chemicals, such as environmental contaminants, food additives, and drugs. It ranges from reduced resistance to infection and neoplasia to allergic and autoimmune conditions. For many years, to identify such immunotoxici chemicals depends on animal models. For animal welfare, ethical and scientific reasons there has been a desire to replace in vivo methods with non-animal alternatives (1). The immune system comprises innate and adaptive immunity. Both arms of the immune response function differently and are driven by different population of cells. A variety of intracellular signaling pathways also play roles in innate and adaptive immune responses. Given the complexity of the immune system, it is unlikely that a single in vitro method will cover all immunotoxicants. Therefore, Integrated Approaches to Testing and Assessment (IATA) has been taken for this field. For example, target compounds are tested using various approaches such as the human whole blood cytokine release assay (HWBCRA), lymphocyte proliferation assay, mixed lymphocyte reaction and fluorescent cell chip assay after the evaluation of myelotoxicity (2). There are many in vitro methods aiming at evaluating various aspects of molecular and cellular events in the adverse outcome pathway (AOP) for immunotoxicity. However, no in vitro method is validated formally.

The IL-2 Luc assay was developed as a part of high-throughput screening system which enables to evaluate chemical immunotoxicity. This screening system was named Multi-ImmunoTox assay or MITA (3) (4) (5). The IL-2 Luc assay, using human cell line transfected with luciferase genes under control of the IL-2 promoter, identifies the effect of chemicals on the IL-2 activity in the 2H4 cells in the presence of stimulants (3).

The PRP was assembled and met in February 2019 to review a progress report on the IL-2 Luc assay prepared by the Validation Management Team (VMT). Following the commentary on this work by the PRP, the VMT refined the validation report.

The PRP engaged in follow-up telephone conferences in October and December 2019. With the provision of all of the amended, updated and additional material, including the final VMT report, this PRP Validation Report was prepared.

IL-2 Luc Test Method Definition

The PRP confirmed that the IL-2 Luc assay test method has been fully described in the report of the Validation Management Team (VMT) and in the associated detailed test protocol. During the validation study, the test developer changed their prediction model. A clear definition of the 35% threshold and its reason was explained. The VMT report describes the need for the assay in the current regulatory context (6). Furthermore, a clear rationale for the assay has been given (the rationale for the test method is that drugs and chemicals, environmental contaminants, food additives, and drugs

can target the immune system, resulting in immune dysregulation). It is known that IL-2 exerts pleiotropic actions on CD4+ T cell differentiation via its modulation of cytokine receptor expression. It promotes Th1 differentiation by inducing IL-12Rb2 (and IL-12Rb1), promotes Th2 differentiation by inducing IL-4Ra, inhibits Th17 differentiation by inhibiting gp130 (and IL-6Ra), and drives Treg differentiation by inducing IL-2Ra. IL-2 also potently represses IL-7Ra, which decreases survival signals that normally promote cell survival and memory cell development (7). Therefore, it is reasonable that the test developer focused on the regulation of IL-2 transcription and attempted to construct an AOP of immunotoxicity with transcriptional dysregulation of IL-2 as a central key event. The VMT report mentioned that IL-2 Luc assay as part of development of a broader tier approach to eventually include IL-8 and IL-1β for corresponding to the AOP.

The PRP agreed that the mechanistic basis of the method and how it related to the T-cell specific endpoint also was well described in the VMT report.

Within Laboratory Reproducibility

The PRP agreed that the results which emerged have demonstrated a sufficient degree of within laboratory reproducibility. For achieving such conclusion, the PRP focused on results obtained with the final protocol and prediction model.

A total of 5 coded chemicals (4 T-cell targeting and 1 non T-cell targeting) were evaluated by 3 experimental sets. Based on such assumptions, the success criterion of >80% within laboratory reproducibility was achieved in each of the three participating laboratories (Lab. A: 80.0% (4/5), Lab. B: 100% (5/5), Lab. C: 80.0% (4/5)).

The PRP notes that extensive documentation of within laboratory reproducibility data for the final and all the development phases of the IL-2 Luc assay has been displayed in the VMT report and its appendices. Taken into account, the data lends support to the view that the assay has a sufficient level of reproducibility within laboratories.

Interlaboratory Transferability

The PRP noted that the technical transfer of the IL-2 Luc assay involved training and successful assessment of 3 experiments of 5 test substances (not blinded) by each of the participating laboratories. That work was prior to their approval to participate in the subsequent validation work.

Between Laboratory Reproducibility

With regard to between-laboratory reproducibility, the PRP recognized that the test results gave 80% (20/25) and met the success criterion of >80% between laboratory reproducibility. The number of test chemicals is combined of the Phase I (5) and Phase II (20) studies.

Again, the PRP notes that extensive and transparent documentation of between laboratory reproducibility data for all phases of the IL-2 Luc assay has been displayed in the VMT report and its appendices.

The PRP concluded that the assay demonstrated successful between laboratory reproducibility.

Predictive Capacity

To determine the predictivity of the IL-2 Luc assay, it is crucial to understand the immunotoxic characteristics of chemicals used in the study. The PRP agreed that classification chemicals into those that affect T cell function, i.e., T cell-targeting chemical (TTC) and those that do not directly affect T cell function, i.e., non-T cell-targeting chemicals (NTTC) . The PRP confirmed the rationale for classifying immunotoxic chemicals are clearly discribed in the VMT report.

Demonstration of a test method’s performance should be based on the testing of representative, preferably coded, reference chemicals. The PRP concluded that the validation study used an appropriate level of test chemical coding to ensure fully blinded evaluation. With respect to chemical selection, the PRP confirmed that the criteria for chemical selection were clearly outlined. On the other, it should be noted that there is a question if the number of true negatives (8/25) in the set was big enough or not.

The immunotoxic characteristics of each chemical used in the Phase I and Phase II studies are shown in the VMT report and based on the criteria total 25 chemicals were classified into 16 positives, 8 negatives and 1 unclassified. According to the classification, accuracy is 75% (18/24), specificity is 75% (6/8) and sensitivity is 75% (12/16). The PRP concluded the predictive capacity of the test was not sufficient, if one would see this test as a stand-alone to detect immunotoxicity.

The PRP basically agreed with the test developer's opinion that there are at least 2 reasons for this poor predictivity. First, the reliability of the criteria about the immunotoxicological chemicals is sometimes uncertain, because the information available was very limited. Second, the IL-2 Luc assay does not cover every aspect of the effects of chemicals on T cell function.

Regarding the second point, the PRP noted again this assay should be used in the context of IATA (that means combination with other assays targeting T-cell functions should be mandatory). With respect to the first point, the PRP noted the use of compounds with clear immunotoxicity mechanism will help to ultimately improve the accuracy of the validation study and, also recommends a Detailed Review Paper (DRP) on the immunotoxicity should stress the importance of reliability of in vivo data .

Following phases I and II, the assay was then applied to over 60 chemicals that were previously evaluated by the test developer (8). The accuracy was calculated as 76%, which is in line with results obtained in phases I and II. As a result, the PRP concluded the predictive capacity of this assay is reasonable if at least the two issues pointed above would be considered.

Applicability Domain

The PRP shared the applicability domain of this assay communication with VMT. Especially because of the use of a cell line, the method can't detect immunotoxicity associated with the inhibition of DNA synthesis and cell division. Another limitation is that the assay might not detect compounds that require metabolic activation to a toxic intermediate. In addition, the use of PMA/Io as stimulants bypasses signaling through the T cell receptor and therefore this stimulation could affect the results. Inevitably, the IL-2 Luc assay shares limitations common to many suspension cell-based techniques, not least in dealing with highly hydrophobic substances.

Performance Standards

The PRP was of the view that the list of performance standard (PS) substances placed in the appendix 15 to the VMT report was satisfactory. Mutual Acceptance of Data (MAD) will only be guaranteed for test methods validated according to the PS, if these test methods have been reviewed and included in this Test Guideline by the OECD. The PS is supplemented by a list of proficiency chemicals, listed in the appendix 14, to be used as a routine check on performance of the assay.

Additional Comments

The PRP concluded that the validation study management and conduct met the criteria set out in OECD GD 34 (2005). The PRP concluded also that the study was conducted not under GLP certification but in the spirit of GLP.

The PRP appreciated the transparency with which all the IL-2 Luc assay material was presented. The PRP notes that during the conduct of the review, it was possible access to the full raw data files associated with the IL-2 Luc assay development/validation work.

The PRP also noted that AOP networks and DRP in this field must be essential in order to construct the IATA.

Conclusions and Recommendations

The PRP concluded that the IL-2 Luc assay validation has demonstrated that the method should be acceptable as part of IATA for the predictive screening of T-cell targeted immunotoxicity.

Acknowledgements

The PRP is grateful to the members of the VMT for their hard work and patience and to JaCVAM for their support in setting up and hosting meetings in Japan, as well as for the setting up of several telephone conferences.

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Teleconference for IL-2 PRP

October 1, 2019

Peer Review Panel: Henk van Loveren, Haley Neff-LaFord, Barbara Kaplan, Fujio Kayama, Takao Ashikaga
VMT: Hajime Kojima
Observers: Steve Venti (meeting minutes)

Kojima:	In this meeting, we will discuss the revised validation report and the schedule going forward. I will explain the changes in the report, which are shown in red. One important point is Appendix 7. It has 290 pages and discusses the data available on immunotoxic effects of chemicals. Mainly, the figures for predictivity and the summary were revised. I heard Dr. Aiba is on-going to revise minorly. After the meeting, I ill share the newest Validation report.
Kaplan:	This summary is in line with what we discussed at the FTF meeting.
Kojima:	Does everyone accept this summary?
Everyone:	Yes.
Kojima:	Section 9-1-3 addresses predictivity and describes the effects of chemicals on T-cells. And there is a definition of T-cell targeting chemicals (TTCs).
Kaplan:	Criterion 3 says “#2 or #3 on two or more cytokines.” Does that refer only to the three cytokines mentioned in #2 and #3? For example, is IL-17 excluded? This is not clear. If there is a report for other cytokines, would they be considered TTCs?
Kojima:	I can’t answer at the moment, but I will ask Dr. Aiba.
Kaplan:	This is an improvement over the original report. Once we have some clarification on Criterion 3, I think that these criteria are acceptable.
van Loveren:	Although I think it would be good to extend this to other cytokines, not just the ones listed.
Kojima:	(Brief review of other changes in red. Please see revised Validation Study Report.) If you are happy with this report, then we can move on to reviewing the PRP Evaluation Criteria and creating the PRP report.
Kaplan:	Do we need to read this and provide comments? What do you need from the PRP to submit to the OECD?
Kojima:	If you feel that the Validation Study Report satisfies the 14 PRP Evaluation Criteria, then you can prepare a Peer Review Report of about 12 pages with a comment about each criterion. And then the Validation Study Report and the Peer Review Report will be reviewed by an OECD expert working group.
van Loveren:	Are there specific places we should comment on?
Kojima:	We revised the Validation Report based on the comments from the PRP.
Kaplan:	So we have already covered the critical issues. But if there is anything specific you want us to look at, please tell us now.
van Loveren:	Is there any issue we need to address now?
Kojima:	I will share these documents with you, and after we have your comments, Dr. Kayama will write the final PRP report.
Neff-LaFord:	Once you see the documents, it is pretty easy to follow what has been changed, so we should be able to follow it.
Kojima:	The deadline for comments if possible, would be by the end of October and then we can have another teleconference in early or mid-November. OK, I will send you meeting minutes, the newest validation Study Report, and the evaluation

	criteria.

Teleconference for IL-2 PRP

November 11, 2019

Peer Review Panel: Henk van Loveren, Barbara Kaplan, Haley Neff-LaFord, Fujio Kayama, Takao Ashikaga, Lin Shi, Xingchao Geng
VMT: Hajime Kojima, Setsuya Aiba, Takuya Kimura
Observers: Steve Venti (meeting minutes)

Kojima:	In this meeting, we will discuss the revised validation report prior to discuss the peer review items. We revised the report based on your comments. After the previous teleconference, we received it in accordance with the comments from Barbara, and you have some other comments that have not been reflected yet, so I think we need to discuss this report more.
Kaplan:	I think these revisions are fine as long as things are separated into a table on criteria and clearly intelligible.
Aiba:	I don't know who made this table, but it presents what I wanted to say, so I think we can use this if the PRP agrees.
Kojima:	Dr. Aiba will calculate predictive capacity based on this table, so the most important thing is that the PRP finds this table acceptable.
Kayama:	I think these criteria are easier to understand as presented in the table.
van Loveren:	I am still concerned that the introduction is confusing to a naïve reader. We of the PRP understand that MITA is the context, *not* the aim, of this study. But the introduction needs a clear statement at the start of the introduction that the aim of this validation study is the IL-2, not MITA in general. Mentioning MITA in the introduction is fine, but you cannot have MITA at the start of the introduction. The introduction must begin with the aim of the study, which is IL-2.
Kaplan:	The first time I read this introduction, I thought that you were validating the entire MITA, but later I realized that is not the case. The goal is to validate the IL-2 assay. I agree with Henk and Haley that the goal of the validation needs to be stated clearly at the start of the introduction. Even just one sentence is enough. Just clearly state that the goal is to validate the IL-2 assay.
Neff-LaFord:	Yes, just more section 3 up higher.
van Loveren:	We need to say "proposed AOP" because this AOP has not yet been accepted.
Neff-LaFord:	The expression "IL-2 LA" appears to mean the same thing as "IL-2 Luc Assay." If IL-2 LA is intended to mean something different, then this needs to be spelled out more clearly.
Aiba:	Yes, I will clarify that.
van Loveren:	On page seven in introduction, I have suggested a revision, but perhaps the information about the applicability range that I deleted needs to be added back.
Kaplan:	I think that in context, the meaning of "applicability domain" is clear enough to be left in. But the word "however" should be removed for clarity.
van Loveren:	The applicability domain is discussed in the preceding paragraph, so maybe we can just use Haley's suggestion as is.
Kojima:	In section 9-5, I will inform you the detailed records collected in the principles of GLP.
Neff-LaFord:	I don't understand what "almost comparable" means in section 10-3-1.
Kaplan:	Given the emphasis on comparing IL-2 results with the results of other tests, I think that this section needs to be expressed more clearly. I think this information is important, which is why it should be described more clearly.
Ashikaga:	I couldn't find any description about regulatory application in the report.

Aiba:	Do I need to respond to each of these comments one by one?
Ashikaga:	Why is SFO-luciferase activity measured in this assay?
Aiba:	It is automatically measured but it is not necessary for this assay.
Kaplan:	This is related to what we were talking about before. This report contains a lot of information that is only incidentally related to IL-2, which confuses the reader.
Ashikaga:	I could not find a list of proficiency chemicals. Shouldn't the developer submit a list?
Aiba:	Yes. Appendix 14 and 15 have a list of proficiency chemicals.
Kojima:	Are there any other comments?
Xingchao:	I agree with the comments and I think the report is improved.
Lin:	(inaudible)
Aiba:	(inaudible)
van Loveren:	The applicability domain does not seem to be defined anywhere. Where is the definition of the applicability domain? All the information is there, but there is no single clear definition. You could rename 10-6 and start with a simple explanation of the applicability domain.
Kaplan:	This is a good point. We have defined a T-cell target, so we need to say that is what the applicability domain.
Aiba:	OK, I will provide a clear definition of what the applicability domain is.
Kojima:	I will share the minutes of this meeting, and then Dr. Aiba and the VMT will revise the validation report to share with the PRP. Perhaps you can then submit your comments for Dr. Kayama within one month and to be created the PRP report by Dr. Kayama.
Kayama:	The most important comment today is Henk's last comment.
Aiba:	I'd like to ask Dr. Kayama to summarize the PRP comments, because I already answered the original comments. I would like to know what I should respond to.
Kayama:	Will the PRP report be incorporated into the validation report or separately attached?
Kojima:	Separately attached.

Teleconference for DRP on in vitro immunotoxicity

October 28, 2019

Emanuela Corsini, Erwin Roggen, Dori Germolec, Henk van Loveren, Barbara Kaplan, Setsuya Aiba, Yutaka Kimura, Takayuki Yoshimoto, Hajime Kojima, Steve Venti (meeting minutes)

Kojima:	Today's agenda is as follows: Opening comments What is DRP in the OECD TG programme? Japanese SPSF adopted at WNT Supporters Discussion of Table of Contents We will allocate responsibilities for the table of contents. I would like to follow Emanuela's suggestion. Do you all agree with this?
Kaplan:	Yes, I will do III.
Germolec:	I can do IV.
Kojima:	I talked with Dr. Aiba and he will do X.
Loveren:	I am fine with II, although I will need some guidance about what to do.
Kojima:	I will do IV, but I have not set I, VI, VII, VIII, or XI. So do we have any ideas for people who could do these sections. The deadline is the end of this year, and then I will share everything by mid-January to prepare for our meeting at the end of January.
Corsini:	Can you explain why these two documents you sent us will help us?
Kojima:	Here is the draft guideline for detection of reproductive and developmental toxicity for human pharmaceuticals. This contains a list of reference compounds based on MOA. I think we need to discuss and select which chemicals we want to discuss.
Corsini:	So you feel these are relevant for immunotoxicity?
Kojima:	We will have to recommend positive and negative compounds for immunotoxicity, so this is a good example for us to follow. But in addition to pharmaceuticals, we have to also address industrial and other chemicals.
Corsini:	So we can use this as a possible template for developing our approach to immunotoxicity.
Aiba:	It's very difficult to determine immunotoxicity just by in vitro tests, because they do not correlate strongly with in vivo test results. So how can we approach this issue? Even chemicals that are classified as immunotoxic do not necessarily increase susceptibility to infection.
Germolec:	I think the goal should be to achieve similar results to in vivo test results for compounds that we know are immunotoxic.
Kojima:	The goal of this document is to encourage the development of in vitro assays for immunotoxicity.
Corsini:	There is no single test that can predict immunotoxicity. So we must always correlate the results of several tests. And the definition will be very broad, because it will encompass any change in functionality of the immune system.
Kojima:	This paper on the Curated Database of Rodent Uterotropic Bioactivity is also an example of an approach we can take.
Corsini:	Yes, we must think of a way to transpose this approach to fit immuotox purposes, but if I understand correctly, these documents are examples that we can follow in preparing our DRP.

Kojima:	Yes.
Germolec:	The information in the literature is rather spotty for trying to see if a chemical was targeting T-cells. So it is difficult to classify what the immunotoxicity would be in vivo. We can try to develop a table like this, but I am not sure it will be useful for all chemicals.
Kaplan:	A lot of work has already been done, so I hope we don't try to reinvent the wheel. We should try to build on what has already been done.
Corsini:	There are many compounds for which we don't have a clear picture of whether they actually target T-cells or not.
Kojima:	I feel that VIII and IX are rather related, so perhaps Dori could make a draft of both these sections.
Germolec:	Yes, I can do VIII and IX. Maybe I will come back to Drs. Kojima and Aiba to ask about how chemicals were selected for the first round of the validation.
Kojima:	Yes, in XI we will be recommending Dr. Aiba's assays. So the validation reports describes much information that can be used as a reference.
Germolec:	Before I send my draft to anyone else, I will send it to Dr. Aiba together with any questions I might have, so that he can confirm that I have covered everything that needs to be covered.
Corsini:	The draft of the introduction can be very brief, because we really all are working independently until we all share our drafts and begin to share comments. So if Hajime can circulate these documents to us then we can all be on the same page about what the introduction should say.
Kaplan:	I agree. Once we see sections I to X, then we will know how to introduce the subject.
Corsini:	Yes, it will be easier to do this at the face to face meeting. So we will submit our drafts by December?
Kojima:	Yes, and then I will distribute them by mid-January, so we can discuss them face to face at the end of January. I will submit to the OECD this May, and if it is approved, then we can discuss our future schedule at the Expert meeting in OECD.

F2F Meeting for the OECD DRP on in vitro immunotoxicity

January 28 & 29, 2020

H. Loveren, B. Kaplan, H. Neff-LaFord, T. Yoshimoto, E. Corsini, D. Germolec, S. Aiba, Y. Kimura, H. Kojima, S. Venti

	January 28
Kojima:	(OECD follow-up activities) SPSF was submitted a year and half ago and was approved in Feb. 2019. Japan is to coordinate creation of a draft DRP. The OECD Secretariat wants to coordinate an expert workshop during 2020 but after the DRP is available.
Corsini:	Does this mean that we have more time than just the end of March to complete the DRP?
Kojima:	We need to identify our action items by the end of this meeting, but we are all busy so maybe the end of March is not possible. Perhaps by the beginning of summer break is a good target.
Corsini:	We have most of the contributions, but we still lack the performance factors. So, we need to ask Erwin to provide this.
Kaplan:	We need to define a difference between IL2-Luc and IL2-LA and when to use.
Germolec:	Is it fair to expect Erwin to contribute? I think his focus is on his family, so we need a contingency in case he cannot write the section on performance factors.
Corsini:	What should be described in Chapter VI?
Germolec:	And how does Chapter VI differ from Chapter VII?
Kojima:	Chapter VII is assay qualification information, which refers to within- and between-laboratory replicability. But the performance factors of Chapter VI refer to test validation criteria.
Aiba:	One issue is that we often don't have a consensus about whether or not a particular chemical is immunotoxic. To create an immunotoxicity test, we need some kind of definition of what is an immunotoxic chemical.
Corsini:	If a chemical presents the possibility of immuno-augmentation or suppression, then we should consider it immunotoxic.
Germolec:	I think that we have pretty strong evidence for many chemicals that they are immunotoxic, so I cannot agree with Dr. Aiba.
Aiba:	This handout is one example of the kind of data we use to determine if a chemical is immunotoxic.
Germolec:	The WHO and the EPA have issued documents that define what makes a compound immunotoxic.
Kaplan:	Are these performance factors specific to IL-2 or are they for all in vitro assays? For all in vitro assays.

Corsini:	We should use the WHO/EPA criteria for classifying compounds immunotoxic. We will adapt the text from the ICHS5 for sections VI and VII.
Loveren:	My understanding is that we need to describe these things at a very general level. VI about is how to arrive at a valid test result, VII is about how to validate the test method itself.
Kaplan:	Do we also need to address issues related to applicability domain? For example, chemicals that require metabolism cannot be identified by an in vitro test.
Corsini:	An in vitro test method should be able to distinguish whether a chemical positive or negative.
Germolec:	It is true that we do not have as extensive a database of chemicals that affect the immune system as we do for chemicals that are sensitizers.
Corsini:	Not all chemicals need to be tested for immunotoxicity, which is another reason that we don't have as much data as for sensitizers.
Germolec:	There are many chemicals I left off my list of immunotoxic chemicals, because I focused on chemicals that would be useful for developing an in vitro test method. Maybe this document is too focused and needs to be made more general.
Corsini:	I received a suggestion to move the background to the start, so that it comes before the introduction.
Loveren:	Yes, and since there is a lot of overlap, I can make the introduction a bit shorter.
Germolec:	Perhaps after having a general explanation of in vitro assays or immunotoxicity, we should have a section at the end of the document using the MITA as an example of how a battery of tests for immunotoxicity is a good approach for in vitro assays.
Aiba:	I don't mind if you delete my introduction and just have your background, because they do describe similar things.
Loveren:	But we do need both sections, don't we? I'm still not 100% sure of how to revise my section. How broad is this? Apparently, we want to be very broad.
Kaplan:	What we can do is provide a broad introduction but then say that we will present the MITA as a possible implementation at the end of the document.
Germolec:	There will be some duplication across sections that we will either have to eliminate or make sure is consistent.
Neff-LaFord:	We are all working on our own sections, but could we share our revisions in real time so that we can each see what has changed?
Kojima:	Maybe we can use the JaCVAM website to share the latest version of the document. I can give everyone a username and password.
Corsini:	What was the source of the text in Chapter IV?
Kojima:	From the AOP wiki
Kaplan:	The OECD project numbers need to be coordinated with AOP numbers so that we know how they relate to the numbers that the OECD secretariat uses. The figures that shows the

	structure of the AOP is much more informative than the current text. Also, the references to “Pending” or “under development” are difficult to understand.
Loveren:	Do we have diagrams for all these AOP?
Kojima:	No, some are under development and there is no diagram.
Germolec:	We should provide links to information that comes from the AOP wiki.
Loveren:	Why is there information on the sensitization AOP? Is it relevant to our DRP? Doesn’t it create confusion to talk about sensitization in the middle of a DRP on immunotoxicity?
Corsini:	Maybe we can just refer to it rather than providing so much information. Immunotoxicity does encompasses skin sensitization.
Kaplan:	Since this is the only AOP that is currently approved by the OECD, perhaps this is a good example of the state of the art.
Germolec:	It might be thought of the type of event that might be found in an immune response AOP. Hypersensitivity is one of the clusters that we refer to when assessing immunotoxicity, so it might be relevant in that sense.
Aiba:	The IL1 AOP is now under EAGMAST review.
Germolec:	The IL1 AOP is a good example of what an immunosuppression AOP should look like, so I would like to see a diagram of it included.
Corsini:	Let’s use the same layout as in Dr. Kimura’s diagram, so that we know the OECD Project No. and status, and include a diagram if available. The AOP chapter needs to be linked with MITA as an example. The next section on the state of the art of in vitro or nonanimal testing, there is still much that needs to be added, which I will do.
Loveren:	But the information on predictive capacity can also be misleading. Because it depends on what chemicals were chosen.
Germolec:	But I think it is valuable to expand this section to discuss these and various other considerations.
Aiba:	There are few drugs that cause immunosuppression by affecting B-cell function.
Neff-LaFord:	There are pharmaceuticals but they are very targeted. There are few environmental chemicals that do that, however.
Aiba:	Our goal is to develop an immunotoxicity test that does not need primary animal cells.
Corsini:	The message that we want to put here is that there are systems that we want to develop into an in vitro test that incorporates T-cell dependent antibody development.
Kaplan:	Should we mention the IL-2 Luc at the end of this chapter or wait until the end? Maybe we can mention that we will talk about the IL-2 Luc in detail at the end of the paper. What are the two assays you mentioned?
Germolec:	The human whole blood and IL-2 Luc assays.

Kaplan:	These two in vitro TDAR assays and the antigen presentation assay are the wave of the future in that they are moving away from using animals, so maybe we can expand on that a little.
Aiba:	Are there any studies that examine cytotoxicity in chemicals rather than drugs?
Corsini:	There are some, but many examine drugs.
Aiba:	The IL-2 test needs to be combined with a test for cytotoxicity. So, what I want to know is if there is a study that shows immunosuppression by suppressing T-cell proliferation.
Kaplan:	Can this discussion of cytotoxicity be incorporated into the performance factors of this paper? I think that this is critical to determining if something is immunotoxic.
Kojima:	This ICHS5 document provides information on what is meant by performance factors and qualification information. So, we can use this document as a reference.
Corsini:	You are suggesting that we adopt this information for our paper? Maybe we can have a flow chart to propose an integrated testing strategy.
Aiba:	We should comment that the IL1-Luc test covers only a narrow range of immunotoxicity, so it cannot achieve a predictivity as high as 80%. We need multiples tests to do that.
Corsini:	Why did you include this sentence saying that "if the proposed test method is to be used in a single laboratory only, multi-laboratory testing will not be required for validation?"
Kojima:	There is need to promote the development of new test methods, so this was included to make it easier for companies to develop in-house assays.
Corsini:	But then the assay cannot be used in a regulatory context, so there is no need for the OECD to review it.
Kojima:	This comes from TG 439 for the development of me-too assays.
Aiba:	The context is different. Our DRP is about the development of a new test method, not a derivation of an already validated one.
Loveren:	Well, we could include that information and keep this sentence.
Corsini:	I think it would be better to delete this sentence.
Kojima:	My idea is to test at least 10 chemicals and achieve a within-laboratory reproducibility of 80%.
Aiba:	My experience is that even just 5 chemicals is enough. 10 chemicals require 90 runs.
Corsini:	It is a statistical issue. It could be 5, it could be 10. Let's leave it at ten. We will get comments from member countries.
Aiba:	Let's make it 5 and get comments.
Corsini:	Let's say "at least 5" but leave it to the agencies and statisticians to create a consensus.
Kojima:	And predictive capacity?
Corsini:	We need to add a phrase to provide context, predictivity might be lower than 80% for a particular assay, but within the context of using multiple assays, that will be acceptable.

Germolec & Loveren:	At the end of this paragraph, let's add this: "It is understood that due to the complexity of the immune system, a highly specific individual test might not achieve the desired levels of specificity, sensitivity, and accuracy, because of the different modes of action of the test chemicals. This can be overcome with a battery of tests, but the battery of tests must achieve the specified predictive capacity.
Corsini:	We also need to provide an explanation as to why some chemicals are excluded from the applicability domain.
Germolec:	We don't have data yet to support an assertion that using a battery of tests will improve predictive capacity. That is our hope, and theoretically we expect this to happen, but we don't have the data to assert this. Perhaps we have to say "We expect that increasing the number of tests will increase predictivity."
Kaplan:	But can we really say that yet?
Germolec:	Yes, it is ok for only one test to give positive results because they all test different things.
Kaplan:	Which means we cannot classify compounds as negative. Only as positive.
Germolec:	The Luster data shows that you need multiple in vivo tests, so based on that we can say that you would need multiple in vitro tests.
Germolec:	I will expand our list of chemicals by adding the ones that I consider relevant and will have to add some negatives, too.
Corsini:	Please add the CAS No. in the table, since this will be a reference.
Germolec:	And I can add some reference works as a footnote to the table.
Loveren:	Do we need to provide chemical selection criteria? Or does it not make so much difference? But anyway, we do need to include negative chemicals.
Germolec:	Please send me any suggestions you have over the next couple of weeks, and I will circulate the new list when it is ready.
Aiba:	Should we suggest that in vitro tests not be performed at concentrations beyond normal human exposure?
Corsini:	We should comment that if an effect is found at a very high concentration, it needs to correlate with in vivo exposure to be considered relevant.

	January 29
Corsini:	Do we want to move some of the discussion in IX to State of the Art and focus here on MITA and other tiered or battery approaches? For example, Lessons from Rodents could be moved.
Kaplan:	I think we can move 2 and 3 up to State of the Art.
Germolec:	We should add an introduction here to in vitro tiered approaches, giving some history. And do we want to move that from where it is now or simply repeat it for emphasis.

Corsini:	I will integrate 2 and 3 into State of the Art, and then add an introduction here to in vitro tiered approach.
Neff-LaFord:	But we will also need to add a conclusion.
Corsini:	Yes, but we will write a discussion and conclusion after we have finalized the content we have now.
Loveren:	Is there really a need to discuss the in vivo tiered approach in this paper? I don't think it has a real bearing on what we are doing here.
Germolec:	I think having that background here establishes a rationale for using the tiered approach for the immune system.
Corsini:	The benefits of a tiered approach are obvious to some, but there are other who are less familiar.
Kaplan:	We don't what to give too much detail about in vivo, just use it to provide a rationale for why a tiered approach is also valid for in vitro testing.
Germolec:	Should the reference chemical section come after Section IX? That way we introduce the MITA and then provide a list of chemicals that can be used to validate any in vitro assay.
Germolec:	We need to remove the "non-immunomodulatory" from headings, because that is misleading.
Kaplan:	We need to be clear in this section as well that the MITA is IL-2 and interferons, but the modified MITA includes IL-8. So, we need to explain clearly what is MITA, what is modified MITA, and where the clusters come from.
Germolec:	What I am struggling with is that we have these clusters, but we don't have a clear concept of what each cluster means for predicting immunotoxicity. Does it mean potency, does it mean mechanism?
Aiba:	They are grouped per their response in the assays.
Germolec:	OK, but if I test an unknown compound and its response in the assays resembles formaldehyde, what does that mean? That is the piece we are missing. We need to include some characterization of the immunosuppressive effect of each cluster.
Corsini:	What we have here is a way of showing that a chemical is, for example, both an immunosuppressive and a skin sensitizer. But we need to spell out for the reader what each cluster represents.
Neff-LaFord:	Yes, use the figure showing the hierarchical clusters but add a table that explains the characteristics of each cluster. But the problem is that we also need to clarify that we are now introducing the element of sensitization, whereas the focus until now has been on immunomodulation.
Aiba:	I will make that table.
Germolec:	We should link the IL-8 Luc assay to the sensitization AOP so that that we can tie them together in this section.

Kaplan:	There seems to be a focus on MITA being IL-2 when actually it includes three cell line and four endpoints.
Neff-LaFord:	If LA refers luciferase activity, then that should be spelled out so that it is less confusing. And we are talking about both MITA and modified MITA in this paper, so we need to clarify that throughout.
Corsini:	Are there any knowledge gaps here that we need to address?
Germolec:	Do we need to propose activities leading to the establishment of a defined approach?
Neff-LaFord:	Do we need to address chemicals that present immunoaugmentation in these assays?
Germolec:	What about expanding the number of chemicals with published test results from a tiered approach? How about the applicability domain and what the limitations are?
Aiba:	We might have to discuss the administration or concentrations, which can also affect the interpretation of results.
Loveren:	That is a consideration but not a data gap.
Germolec:	We might need to distinguish here between key considerations and data gaps. How does a change in cytokine expression translate into health risk assessment? So perhaps we need to call out in-vivo–in-vitro extrapolation, and also mention that it is for all in vitro studies, not just immunotoxicity. And then we need a conclusion, that might be a place to discuss the use of multiple assays to test different aspects of immunotoxicity.
Corsini:	And say that much progress has been made in the field of immunosuppression using non-animal approaches.
	Schedule
	Revisions by end of February
	Collated document distributed before SOT
	Additional comments by end of March
	Conference call in April before WNT
	Present document to WNT at end of April
	Conference call in May
	Final document by end of June

Expert Group on DASS

Teleconferences 18/19 November, 2019 14h30-17h (Central Europe)

Attendees
18 November: Susanne Kolle, Donna Macmillan, Chantra Eskes, Knud Ladegaard Pedersen, Amaia Irizar, Nathalie Printemps, Michele Regimbald-Krnel, Silvia Casati, Erika Witasp Henriksson, Andrea Gissi, Tim Singer, Grace Patlewicz, Andre Muller, Raja Settivari, Henrik Tyle, Elena, Eva Bay Wedebye, Martin Paparella, Sebastian Dunst, Judy Strickland, Kristie Sullivan, Nicole Kleinstreuer, Petra Kern, Roman Liska, LMC, Martina Klaric, Nathalie Alepee, Matthias Herzler, Andreas Natsch, Paul Brown, Janine Ezendam, Andrew Williams, Emma Grange, Hermann-Josef Thierse, Laura Rossi, Gavin Maxwell, Anne Gourmelon, Patience Browne

November 19: Kristie Sullivan, Michele Regimbald-Krnel, Takao Ashikaga, Andrea Gissi, Silvia Casati, Chantra Eskes, Donna Macmillan, Janine Ezendam, Judy Strickland, Tim Singer, Grace Patlewicz, Petra Kern, Nicole Kleinstreuer, Nathalie Alepee, Andreas Natsch, Betty Hakkert, Gavin Maxwell, Matthias Herzler, Ovanes Mekenyan, Sebastian Dunst, Emma Grange, Martin Paparella, Laura Rossi, Hermann-Josef Thierse, David Asturiol, Erika Witasp Henriksson, Andrew Williams, Amaia Irizar, Jong Kwon Lee, Eva Bay Wedebye, LMC, Susanne Kolle, JaCVAM, Andre Muller, Nathalie Printemps, Andrea Gissi, Gavin Maxwell, Anne Gourmelon, Patience Browne

DAY 1

1. Discussion of LLNA and Human reference data
    a. Matthias Herzler (BfR) presented an evaluation of uncertainty in LLNA data
        i. Table 4 summarised reference chemical classifications for
            1. binary calls (GHS_{BIN}),
            2. GHS subcategory classification of potency (GHS_{SUB}), and
            3. using the new proposed approach, two additional classifications for borderline 1A/1B chemicals =1 and 1B/NC chemicals (GHS_{BORDER}).
        ii. In most cases (86/108 chemicals), the analysis from the LLNA data subgroup (using the Median-Like Location Parameter (Hoffmann et al. 2018)) and this new approach produced consistent results for LLNA reference chemical GHS classifications.
        iii. For 17 chemicals, previous classifications were proposed to be changed to "ambiguous" calls (1 or NC/1B).
        iv. The EG discussed the change to calls, specifically
            1. Changes from NC to 1B/NC would reduce the number of negative reference chemicals to 11
            2. The change in potency calls for some reference chemicals was based on rejecting negative results with maximum test chemical concentrations of $\leq$25%
            3. Feedback was provided, that while few chemicals may be tested at higher concentrations, it is rare.
        v. A calculation of the LLNA reproducibility based on the number of studies available per individual substance and removing the ambiguous results increases the reported value considerably (~95%).
    b. The JRC presented an overview of ambiguous LLNA reference chemical classifications in the LLNA data subgroup database supporting the revised GL and SD
        i. The presentation included some feedback on specific chemicals received following the 1 November commenting round
        ii. As with Matthias's presentation, for many chemicals, understanding the data required a deeper examination of the LLNA studies

c. The US presented an overview of ambiguous human reference chemical classification from the human data reference subgroup report and database
    i. The presentation also included feedback on specific chemicals received following the 1 November commenting round
    ii. Several experts commented that human data could not be reliably used for potency but a comparison for hazard assessment should be included in the SD (and summarised in GL)
d. Following some discussion of individual chemicals, **a decision was taken to reflect on the analyses and provide input on a final reference chemical list by 26 November** to:
    i. address questions regarding specific chemical data, and
    ii. propose an approach for dealing with borderline chemicals

DAY 2 – addition topics for discussion in presentation from US

2. The group discussed the status of the 2 out of 3 DA in the draft GL
    a. As stated in the email from Anna Lowit (18November 2019), the US is accepting the DA in lieu of LLNA data for hazard classification
    b. Several members of the DASS EG noted the high FN rate and the questionable relevance of the 2 out of 3 DA for negative calls
        i. While it was noted that the DA predictions may change a bit with updated reference chemical data, the FN rate is not expected to change dramatically
        ii. It was also noted that the mispredictions often involve chemicals with borderline LLNA results
    c. The US presented several options for the 2 out of 3 DA moving forward
    d. DK remarked that there is support for moving the ITS forward at this time but the 2 out of 3 may benefit from additional analyses
3. The versions of the ITS DA were discussed
    a. Several comments remarked that it was unclear if the ITS v1 (using Derek) and v2 (using OECD QSAR TB) should be one or two DAs.
    b. There was general agreement that these should be two versions of one DA
        i. Donna Macmillan/UK expressed a dissenting opinion
    c. The text proposed by DK to describe the change in the DIP for the ITS was supported with a modification to explain the rationale of the revised cutoff to 6 (due to the change between ECETOC and GHS potency classification systems and resulting in better prediction of 1A chemicals, etc.)
4. Substitute in vitro and in silico data in DAs
    a. The EG supported including more details on assumptions made and protocol to produce in silico predictions in GL (e.g. in an annex)
    b. More details are needed in the text proposed by the UK/LMC regarding “sufficiently reliable” commercial in silico tools (in addition to Derek and OECD TB)
        i. The group supported reversion to previous version with more details regarding publications, QMRF, etc.
        ii. The following text is proposed “The ITS may use other reliable in silico tools which are well supported by publications, have QMRF with complete information for the model, such as training set, applicability domain, performance, etc. with documentation equivalent to the in silico tools included in the GL.”
            1. This will be further refined between the leads/Secretariat
        iii. DK suggests that similar language be crafted for other in vitro information sources.
5. Comments on applicability domain
    a. More guidance may be needed on how to use metabolism/reactivity domain profilers – these are not intended to be used as QSARs and may need to be validated

b. Comments received regarding applicability domain were discussed regarding more/less prescriptive information on the applicability domain (Figure 1 from GL)
    i. EG suggested framing the analysis as a description of the current dataset rather than an interpreting the figure as prescriptive
    ii. Suggestion to remove statements recommending methods/DAs as "better" for some chemicals than others, as this only reflects the limited number of chemicals tested in each domain
    iii. Details should be only in the SD, including how reactivity domains were defined for this analysis
    iv. Leads will revise figure to clarify workflow and associated text
c. Add text to GL specifying that mixtures/UVBCs, (and in this case) metal, and inorganic chemicals are outside the applicability domain

6. Responses to comments on me-too in vitro information sources
    a. DK suggested "parking" the issue for now and having a broader discussion at the WNT
        i. Support for adding the topic to the 2020 WNT meeting
    b. Topic is probably easier to address in the hazard identification mode than for potency
        i. For ITS with quantitative cutoffs, they are assay specific and cannot be extended to a drop-in replacement
    c. As noted in the discussion of in silico, language could be included to suggest other options may be acceptable
7. Next steps
    a. EG will have an additional chance to provide feedback on the LLNA and human reference chemicals (circulated in September) and the analysis from Matthias and provide additional chemical-specific consideration until 26 Nov
    b. TC scheduled for 1st week in December to resolve final reference chemicals

厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）

OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

令和元年度 分担研究報告書

発がん性試験のTGおよび発がん性AOP開発

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研究要旨

毎年、国内外で多くの新規化学物質が考案され、我々の日常生活に応用されている。一方、化学物質の発がん性評価は 1 群雌雄 50 匹で 3 用量及び対照群を設けたげっ歯類を用いた 2 年間反復投与試験がスタンダードとされており、時間的、コスト的およびヒトへの外挿性の観点からも、その実施は限定的となっている。本研究では、より効率的な発がん性試験法の TG 開発及び発がん機序に基づく発がん性評価法の基礎となる AOP の開発を主な目的とするとともに、現在 OECD で作成が進められている非遺伝毒性発がん性の IATA 作成に対面会議や電話会議を通じて発がんの専門家としての協力が求められる。

1990～2000 年代に名古屋市立大学のグループで開発され、既に厚生労働省の化学物質発がん性スクリーニングに採用されているラット肝中期発がん性試験について、前年度に OECD に提出した SPSF (Standard Project Submission Form) に対する質問事項に対応した。TG 提案への同意は得られなかったが、本法の有意性について、テストガイドライン作業グループ会合において紹介した。

さらに日本製薬工業会グループ（研究協力者 久田茂博士ほか）の協力を得て、医薬品のラットにおける非遺伝毒性発がん性 AOP に関する SPSF として提案した 13 件の AOP のうち、最もヒトへの外挿性が考えられる「トリプシン阻害による膵腺房細胞腫瘍」に関する AOP について、AOP Wiki に登録した。また、非遺伝毒性発がん機序の一つである細胞障害を key event とする鼻腔発がんの AOP を OECD に提出中であるが、ホルムアルデヒドによる酸化的ストレスの関連文献等の情報を取り纏め、酸化的ストレスを起始分子イベントとする AOP の作成を進めることとした。

また、OECD で進められている非遺伝毒性発がん性の IATA 策定に関する専門家会議の検討方針についての論文作成の議論に参加した。投稿論文は minor revision となった。

A. 研究目的

毎年、国内外で多くの新規化学物質が考案され、我々の日常生活に応用されている。一方、化学物質の発がん性評価は 1 群雌雄 50 匹で 3 用量及び対照群を設けたげっ歯類を用いた 2 年間反復投与試験がスタンダードとされており、時間的、コスト的およびヒトへの外挿性の観点からも、その実施は限定的となっている。さらに、動物実験の 3Rs に対する国際動向に呼応し、動物実験からヒト材料を用いた *in vitro* 試験、あるいは毒性作用機構に基づく安全性評価手法の開発が進められている。OECD においても、反復投与毒性、生殖発生毒性、感作性、発がん性などの毒性発現機構を明確にするために AOP（Adverse Outcome Pathway）を作成し、それらの情報を網羅した IATA（Integrated Approaches to Testing and Assessment）により化学物質の行政的な安全性評価を推進する戦略を進めている。

本研究では、より効率的な発がん性試験法の TG 開発及び発がん機序に基づく発がん性評価法の基礎となる AOP の開発を目的とするとともに、現在 OECD で作成が進められている非遺伝毒性発がん性の IATA 作成に対面会議や電話会議を通じて発がんの専門家としての協力が求められる。

B. 研究方法

B.1. 発がん性試験の TG 開発（小川）

1990～2000 年代に小川が在籍していた名古屋市立大学のグループで開発され、既に厚生労働省の化学物質発がん性スクリーニングに採用されているラット肝中期発がん性試験について、前年度に OECD に提出した SPSF (Standard Project Submission Form) に対する質問事項に対応した。

B.2. 発がん性の AOP 開発（西川・小川）

（1）日本製薬工業会グループ（久田茂博士等）の協力を得て、OECD に提案した「ラットにおける非遺伝毒性発がん性 AOP に関する SPSF」について、OECD の質問事項に対応した。

（2）研究分担者の西川および小川は、ラット，マウスおよびハムスターに鼻腔腫瘍を誘発した化学物質に関連し、ホルムアルデヒドに起因する酸化的ストレスに関する文献検索を実施した。

B.3. 非遺伝毒性発がん性の IATA 作成への協力（西川・小川）

非遺伝毒性発がん性IATA作成専門家会議の検討方針に関する論文作成について、電話会議等で議論に参加した。

（倫理面への配慮）

本研究は動物実験の 3Rs に配慮して試験法の開発を主とするものであり、実験動物，ボランティアおよびヒト組織は使用しないことから、倫理的問題は無いと考える。

C. 研究結果

C.1. 発がん性試験の TG 開発（小川）

ラット肝中期発がん性試験の SPSF に対する OECD 加盟各国からのコメントに対応した回答集及び SPSF の改訂版を作成し OECD に提出した。TG 提案への同意は得られなかったが、本法の有意性について、テストガイドライン作業グループ (Working Group of National Co-ordinators of the TGs programme; WNT) 会合において紹介した。

C.2. 発がん性の AOP 開発（西川・小川）

(1) 前年度に、研究代表者（小島）を介して

OECD に提案し、改訂を行った、13 件のラットにおける非遺伝毒性発がん性 AOP の SPSF について、OECD からコーチの提案を受けた。13 件の AOP のうち、最もヒトへの外挿性が考えられる「トリプシン阻害による膵腺房細胞腫瘍」に関する AOP について、日本製薬工業会のグループとともに作成し、AOP Wiki に登録した。
https://aopwiki.org/aops/316
(2) 前年度の検討において、網羅的に情報収集した鼻腔発がん物質（ラット 41 物質、マウス 5 物質、ハムスター7 物質）によって誘発された鼻腔腫瘍を INHAND に基づいて分類した結果、10 種類の腫瘍性病変（扁平上乳頭腫・癌、腺腫・腺癌、腺扁平上皮癌、神経上皮癌、軟部肉腫等）に分類されたが、鼻腔腫瘍と先行病変と考えられる非腫瘍性病変との関連は癌であっても腫瘍毎に異なる傾向が見られた。AOP 作成のため，鼻腔粘膜の細胞障害を引き起こす起始分子イベントを検索したところ、酸化的ストレスに関連する文献が比較的多く見つかった。ホルムアルデヒドによる酸化的ストレスの関連文献は 2015 年から現在までに 6 件報告されており、AOP の作成において有用な情報となった。

C.3. 非遺伝毒性発がん性の IATA 作成への協力（西川・小川）
OECDで進められている非遺伝毒性発がん性のIATA策定への協力の一環として、専門家会議の検討方針に関する論文作成に関する電話会議に参加した。論文“Chemical carcinogen safety testing: OECD expert group international consensus on the development of an integrated approach for the testing and assessment of chemical non-genotoxic carcinogens” はArchive of toxicology に投稿中である。

D. 考察

D.1. 発がん性試験の TG 開発（小川）
ラット肝中期発がん性試験の OECD の TG 申請は同意が得られなかったが、引き続き非遺伝毒性発がん性の IATA 等への引用を目指し、バリデーション情報等を取り纏めて発信する必要性があると考えられた。

D.2. 発がん性の AOP 開発（西川・小川）
(1) OECD に提案した 13 件のラットにおける非遺伝毒性発がん性 AOP の SPSF のうち、最もヒトへの外挿性が考えられる「トリプシン阻害による膵腺房細胞腫瘍」に関する AOP について AOP Wiki に収載された。他の提案についても、ヒトへの外挿性を考慮しつつ、AOP 化を目指す。
(2) ホルムアルデヒドによる酸化的ストレスの関連文献等の情報を取り纏め、酸化的ストレスを起始分子イベントとする AOP 作成を進めると同時に、鼻腔発がんの病理発生にかかる論文化を目指す。

D.3. 非遺伝毒性発がん性の IATA 作成への協力（西川・小川）
OECDの当該専門家会議の方針に関する論文は受理のめどが立ったため、非遺伝毒性発がん性のIATA作成については、可能な限り協力を続ける。

E. 結論

E.1. 発がん性試験の TG 開発（小川）
ラット肝中期発がん性試験の TG 化は困難であるが、本法の有用性について、引き続き IATA への引用などで貢献する意義があると考えられた。

E.2. 発がん性の AOP 開発（西川・小川）

（１）ラットにおける非遺伝毒性発がん性AOPとして提案した13件のSPSFのうち、1件がAOP Wikiに収載された。

（２）細胞毒性を初動因子とした鼻腔発がんのAOP開発に関しては、酸化的ストレスの関与が重要と考えられた。

E.3. 非遺伝毒性発がん性の IATA 作成への協力（西川・小川）

非遺伝毒性発がん性のIATA作成を継続し、検討方針に関する論文化に貢献した。

F. 研究発表

F.1. 論文発表

本課題に直接関連する論文発表はありません。（minor revision中）

F.2. 学会発表

本課題に直接関連する学会発表はありません。

G. 知的財産権の出願・登録状況

G.1. 特許取得

該当なし

G.2. 実用新案登録

該当なし

G.3. その他

該当なし

H. 添付資料

AOP 316: Trypsin inhibition leading to pancreatic acinar cell tumors

AOP ID and Title:

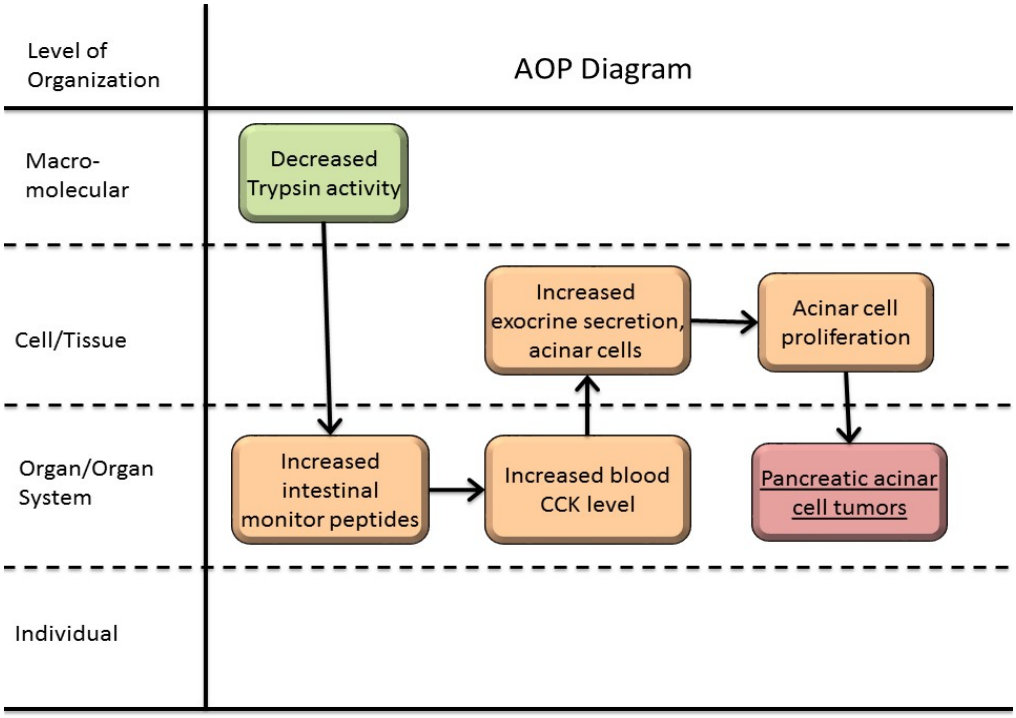
SNAPSHOT

Created at: 2020-01-16 16:52

AOP 316: Trypsin inhibition leading to pancreatic acinar cell tumors

Short Title: TI-induced AC tumors

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.60	Included in OECD Work Plan

Abstract

Pancreatic exocrine secretion is controlled mainly by the gastrointestinal hormone cholecystokinin (CCK), which is secreted by CCK-producing I cells located in the mucosa of the small intestine. Once the contents in the stomach is transported to the small intestine, I cells are stimulated to release CCK into the bloodstream. Several mechanisms to stimulate CCK release are involved.

In rats, pancreatic acinar cells secrete monitor peptide (MP) into the intestinal lumen as a pancreatic soluble trypsin inhibitor (TI). MP stimulates I cells to release CCK into the bloodstream through their surface MP receptors. Then, the increased blood concentration of CCK induces pancreatic exocrine secretion. When fasting, trypsin–MP complexes are formed to decrease the level of free MP in the small intestinal lumen; thereafter, CCK release is suppressed. Meanwhile, upon feeding, partially ingested proteins in the diet consume trypsin to increase the luminal concentration of free MP followed by stimulation of CCK release.

When soybean powder (raw soya flour) containing trypsin inhibitory molecules or TIs such as camostat are given to rats, the intestinal concentration of free MP is increased due to trypsin–TI complex formation. Then, intestinal I cells are stimulated to release CCK. The resulting increased blood level of CCK stimulates pancreatic exocrine secretion of MP, which induces further CCK release via a positive feedback loop. A sustained increase in the CCK level might induce pancreatic hypertrophy and hyperplasia and ultimately result in acinar cell tumor formation.

This increased blood CCK level induced by trypsin inhibition may also occur in humans and other mammalian species including rats. Luminal CCK-releasing factors (LCRFs) are trypsin-sensitive peptides secreted from small intestinal mucosa that stimulate CCK release by intestinal I cells. Luminal levels of LCRFs are increased after TI ingestion; however, the resultant increase in CCK levels does not stimulate further release of LCRFs, in contrast to MP.

Species differences in CCK-mediated stimulation of pancreatic enzyme secretion have been described in rats and humans. In rats, CCK stimulates pancreatic exocrine secretion and/or proliferation directly via CCK1 receptors expressed on acinar cell surfaces or indirectly via vagal afferent nerves expressing CCK1 receptors, especially at physiological blood CCK concentrations. In contrast to rats, the secretory function of human pancreatic acinar cells is indirectly innervated by vagal afferent nerves expressing CCK1 receptors; however, CCK receptors (mainly CCK2 receptors) expressed on human acinar cell surfaces are not involved in both exocrine secretion and proliferation. These findings suggest that, in humans, innervation of acinar cells in response to elevated CCK blood levels affects mainly secretory functions, with less of an effect on cell proliferation, although the effects of vagal stimulation on acinar cell proliferation are still unclear.

In conclusion, long-term administration of TIs induces pancreatic acinar cell tumors in rats. The main factor contributing to carcinogenesis is a sustained increase in plasma CCK levels mediated by an increased luminal concentration of trypsin-sensitive MP. The risk of trypsin inhibition-induced pancreatic tumors in humans seems to be low or equivocal because of the following reasons:

1. MP, a pancreatic soluble TI that protects against auto-injury induced by trypsin, stimulates CCK release and thereby pancreatic exocrine secretions containing MP, via a positive feedback loop, in rats only.
2. An increased CCK level directly stimulates pancreatic acinar cells to proliferate via surface CCK1 receptors in rats but not in humans. It is still unclear whether vagal stimulation of acinar cells promotes proliferation of acinar cells.

Background

Raw soy flour and purified trypsin inhibitors (TI) cause pancreatic hypertrophy and hyperplasia in some mammalian species, and prolonged treatment with high levels of TI contained in raw soy induced pancreatic nodular hyperplasia and acinar cell adenoma [Rackis JJ, 1965; McGuinness EE et al, 1984; McGuinness EE et al, 1980; McGuinness EE et al, 1985; McGuinness EE and Wormsley KG, 1986; Gumbmann MR et al, 1986]. TI also promoted nodular hyperplasia and tumor formation in rats treated with low levels of pancreatic carcinogens such as azaserine [McGuinness EE et al, 1984; McGuinness EE et al, 1987; Lhoste EF et al, 1988]. These findings question the safety of TI-containing plant foods, and many different studies and reviews have been published to date. The important factors for TI-induced pancreatic acinar cell tumors seem to be a high level of CCK release and CCK-stimulated acinar cell proliferation. In the present AOP, the pathway progressing from trypsin inhibition to pancreatic acinar cell tumor formation is considered from the viewpoints of such key factors.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	1720	Trypsin inhibition (https://aopwiki.org/events/1720)	Inhibition, trypsin
	KE	1721	Increased intestinal monitor peptide level (https://aopwiki.org/events/1721)	Increased monitor peptide
	KE	1722	Increased blood CCK level (https://aopwiki.org/events/1722)	Increased blood CCK level
	KE	1723	Increased exocrine secretion from pancreatic acinar cells (https://aopwiki.org/events/1723)	Increased acinar cell exocrine secretion
	KE	1724	Acinar cell proliferation (https://aopwiki.org/events/1724)	Acinar cell proliferation
	AO	1725	Pancreatic acinar cell tumors (https://aopwiki.org/events/1725)	Acinar cell tumors

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Trypsin inhibition (https://aopwiki.org/relationships/2028)	adjacent	Increased intestinal monitor peptide level	Moderate	Low
Increased intestinal monitor peptide level (https://aopwiki.org/relationships/2029)	adjacent	Increased blood CCK level	High	Moderate
Increased blood CCK level (https://aopwiki.org/relationships/2030)	adjacent	Increased exocrine secretion from pancreatic acinar cells	High	High
Increased exocrine secretion from pancreatic acinar cells (https://aopwiki.org/relationships/2031)	adjacent	Acinar cell proliferation	High	Moderate
Acinar cell proliferation (https://aopwiki.org/relationships/2032)	adjacent	Pancreatic acinar cell tumors	High	High

Overall Assessment of the AOP

Long-term treatment with trypsin inhibitors (TIs) such as raw soya flour (RSF) in rats increases the incidence of pancreatic acinar cell tumors [McGuinness EE et al, 1984; Gumbmann MR et al, 1986; McGuinness EE et al, 1987; Woutersen RA et al, 1991]. The causative factors for tumorigenesis are a TI-induced increase in cholecystokinin (CCK) release from small intestinal I cells into the blood and direct stimulation of acinar cell proliferation via surface CCK1 receptors [Watanapa P and Williamson RC, 1993].

Differences in these tumor risk factors between rodents and humans are described below.

In rats, trypsin-sensitive monitor peptide (MP), a pancreatic soluble TI (PSTI) found in pancreatic juice that protects against the auto-injury induced by trypsin [Iwai K et al, 1987; Iwai K et al, 1988; Tsuzuki S et al, 1991; Tsuzuki S et al, 1992], plays a major role in stimulating pancreatic exocrine secretion via CCK release [Miyasaka K et al, 1989; Fushiki T et al, 1989; Miyasaka K and Funakoshi A, 1998]. TIs increase the luminal concentration of MP to stimulate CCK release, which in turn increases the MP level as well as pancreatic enzyme secretion via positive regulation. Moreover, repeated injection of CCK into rats increased the level of MP mRNA in the pancreas [Tsuzuki S et al, 1992]. Therefore, the TI-induced increase in CCK release seems to be robust in rodents compared with other species.

On the other hand, in humans, PSTIs do not directly stimulate CCK release [Miyasaka K et al, 1989]. Furthermore, other trypsin-sensitive CCK-releasing peptides (luminal CCK-releasing factors, LCRFs) secreted by intestinal mucosal cells are found in multiple species including rodents and humans [Spannagel AW et al, 1996; Herzig KH et al, 1996; Tarasova N et al, 1997; Li Y et al, 2000; Owyang C, 1999; Wang Y et al, 2002]. TIs increase luminal concentrations of LCRFs, which stimulate CCK release; however, the increase might be mild compared with that induced by MP, because LCRF release does not increase in response to increased CCK levels.

Regarding mitotic activity, high plasma levels of CCK directly stimulate proliferation of rodent pancreatic acinar cells via their surface CCK1 receptors [Povoski SP et al, 1994; Myer JR et al, 2014]. In humans, surface CCK receptors (mainly CCK2 receptors) are not involved in stimulating pancreatic functions; the secretory functions of human acinar cells are innervated mainly by vagal afferent nerves expressing CCK1 receptors [Dufresne M et al, 2006]. However, the vagal contribution to acinar cell proliferation is controversial. Oral ingestion of raw soya flour containing TIs has been reported to stimulate CCK release in humans [Calam J et al, 1987]. In addition, some epidemiological surveys suggest that long-term ingestion of TI-containing foods does not increase the risk of pancreatic cancer [Miller RV, 1978]. On the other hand, a strong relationship between pancreatic cancer and a history of subtotal gastrectomy [Mack TM et al, 1986], which induced a higher plasma CCK level in response to fat [Hopman WP et al, 1984], was reported.

Therefore, the present AOP supports a pathway from trypsin inhibition to tumor formation originating from pancreatic acinar cells in rodents. The relevance of these findings to humans seems low, although some evidence of a TI-induced increase in blood CCK levels suggests the need for case-by-case risk assessment of pancreatic cancer in humans.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Macaca fascicularis	Macaca fascicularis	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Mus musculus	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Sex Applicability

Sex	Evidence
Mixed	High

Trypsin

Trypsin is a pancreatic digestive enzyme that has been identified in many animals, including insects, fish, and mammals. The natural substrate of trypsin is generally any peptide that contains Lys or Arg. The active site of trypsin, which is composed of a catalytic triad, is fully conserved, with a similar three-dimensional structure among species, although there are species differences in the amino acid sequence of the enzyme [Baird Jr TT and Craik CS, 2013; Baird Jr TT, 2017]. TIs such as soybean flours and camostat suppress the activity of trypsin in animal species, including rats and humans [Savage GP and Morrison SC, 2003].

Monitor peptide and related peptides with trypsin inhibitory activity

Pancreatic soluble trypsin inhibitors (PSTIs) are found in the pancreatic juice of multiple mammalian species, including rodents and humans [Greene LJ et al, 1968; Pubols MH et al, 1974; Eddeland A and Ohlsson K, 1976; Kikuchi N et al, 1985]. Secreted PSTIs bind tightly to trypsin to protect against trypsin-induced self-injury in the pancreas and intestinal tracts [Voet D and Voet JG, 1995].

In rats, two types of PSTIs have been isolated: monitor peptide (MP, also known as PSTI-I) and PSTI-II [Tsuzuki S et al, 1991; Tsuzuki S et al, 1992]. Both are similar in amino acid sequence; however, the former directly stimulates CCK release from intestinal I cells via their surface MP receptors, whereas the latter does not [Yamanishi R et al, 1993]. Human PSTIs do not directly stimulate CCK release from intestinal mucosal cells [Miyasaka K et al, 1989].

Species differences in the mechanism of CCK release

Pancreatic exocrine secretion is controlled mainly by CCK released into the blood steam from intestinal mucosal I cells of the small intestine in response to the gastric contents transported to the intestine [Singer MV and Niebergall-Roth E, 2009; Rehfeld JF, 2017]. Peptides released from gastrointestinal digestion, along with fatty acids, are the main stimuli of CCK release involving several direct and indirect pathways [Caron J et al, 2017].

In humans and canines, amino acids and fatty acids in the gastric contents transported to the small intestine play a major role in stimulating CCK release, which regulates pancreatic exocrine secretion, but MP is not involved in exocrine regulation [Wang BJ and Cui ZJ, 2007].

In rats, however, different from other mammalian species, MP secreted by pancreatic acinar cells plays a major role in protein-stimulated CCK release [Iwai K et al, 1988; Fushiki T et al, 1989]. Ingestion of trypsin inhibitors increases the intestinal level of MP, especially in the intestines during fasting, causing a subsequent increase in the blood level of CCK. Increased levels of CCK stimulate pancreatic exocrine secretion of proteins including MP, which in turn further increases the release of CCK. This positive feedback response associated with MP secretion might lead to continuously elevated plasma levels of CCK [Liddle RA, 1995].

Species differences in CCKs

Several isoforms of CCK, including CCK-83, -58, -39, -33, -22, and -8, have been identified, and there are species differences in CCK isoforms (e.g., CCK-33, -22 and -58 are expressed in humans, CCK-58 in dogs, CCK-8, -33 and -58 in cats, CCK-22, -58, -3 and -8 in pigs, CCK-22 and -8 in rabbits, and CCK-58 in rats). All of these isoforms of CCK have a highly conserved region of amino acids, and all are ligands of CCK1 receptors [Wang BJ and Cui ZJ, 2007].

Species differences in pancreatic exocrine secretion

In rats, physiological plasma level of CCK stimulates pancreatic exocrine secretion and acinar cell growth directly via CCK1 receptors expressed on the cell surface, and exocrine secretion is also innervated by vagal afferent nerves expressing CCK1 receptors [Singer MV and Niebergall-Roth E, 2009; Pandiri AR, 2014]. Higher plasma levels of CCK may stimulate acinar cell proliferation only via surface CCK receptors but not by vagal nerve innervation [Yamamoto M et al, 2003].

On the other hand, human pancreatic acinar cells express CCK2 receptors, which are not involved in secretion nor proliferation, and exocrine secretion is regulated exclusively by innervation of vagal nerves expressing CCK1 receptors [Soudah HC et al, 1992; Beglinger C et al, 1992; Singer MV and Niebergall-Roth E, 2009], although there is some evidence of direct stimulation of exocrine secretion of human pancreatic acinar cells [Murphy JA et al, 2008].

Species differences in CCK receptors

Although the distribution of CCK receptors is different between humans and rodents, the structures of CCK1 receptors are highly conserved among mammalian species, and all CCK isoforms function as ligands of CCK1 receptors [Wang BJ and Cui ZJ, 2007].

In rats, CCK1 receptors are expressed in pancreatic acinar cells and sensory vagal afferent nerves, whereas in humans, CCK1 receptors are expressed in vagal afferent nerves but not pancreatic acinar cells. Acinar cells instead express CCK2 receptors; however, these CCK2 receptors are not involved in pancreatic exocrine secretion [Ji B et al, 2001; Dufresne M et al, 2006].

Risk of TI-induced tumor formation from pancreatic acinar cells in humans

The mode of action of TI-induced tumor formation from pancreatic acinar cells in rats is based on a persistent increase in the blood level of CCK, which is induced by an increased intestinal level of MP, resulting from positive regulation of pancreatic exocrine secretion and TI activity.

It was reported that raw soya flour increases CCK release in humans [Calam J et al, 1987]. In addition, the plasma CCK concentration was found to increase after oral administration of fat in patients after subtotal gastrectomy [Hopman WP et al, 1984], and a strong association between pancreatic cancer and a history of subtotal gastrectomy was demonstrated in these patients [Hopman WP et al, 1984].

Therefore, based on the findings from animal studies of persistently increased blood CCK levels accompanied by histopathologic changes in acinar cell proliferation, the tumor risk should be evaluated carefully in humans, despite the lower risk compared with rodents.

Essentiality of the Key Events

MP:

Atropine-treated rats with diversion of pancreatic juice were infused with a mixture consisting of MP, purified trypsin, and various food proteins into the small intestine after intraluminal lavage, followed by examination of pancreatic exocrine secretion. Exocrine secretion was fully reconstructed by the constituent, suppressed in the absence of MP, and the treatment with an anti-MP antibody decreased this exocrine secretion [Fushiki T et al, 1989]. These results suggest that MP is an essential factor for regulating pancreatic exocrine secretion.

CCK:

CCK-deficient mice generated by gene targeting in embryonic stem cells showed no abnormalities in body weight or pancreatic weight or histopathology, but they showed protein-induced increases in pancreatic growth and proteolytic enzyme secretion, suggesting that other regulatory pathways are modified to compensate for the CCK deficiency [Lacourse KA et al, 1999]. The TI camostat increased pancreatic wet weight and protein and DNA levels in a time-dependent manner over a 10-day period in normal mice, but not in CCK-deficient mice [Tashiro M et al, 2004]. These results suggest that CCK is needed for TI-induced pancreatic hyperplasia.

CCK receptors:

In an experiment in which CCK1 receptor-deficient rats were fed a diet containing 0.1% TI (camostat, ONO-3403, or soybean TI) for 7 days, the CCK mRNA level increased without any change in the protein level in pancreatic juice in each TI treatment group. These results suggest that TI treatment enhances the release of CCK, and that CCK-induced secretion of pancreatic digestive enzymes is mediated by CCK1 receptors [Kawanami T et al, 1999].

Experiments using CCK1-receptor-deficient Otsuka Long-Evans Tokushima Fatty rats showed the following:

1. The CCK1 receptor plays a role in the increased cell size associated with normal growth of the pancreas [Miyasaka K et al, 1996].
2. The CCK1 receptor is not an absolute requirement for normal growth of the pancreas but is important for pancreatic regeneration [Miyasaka K et al, 1997].
3. Absence of the CCK1 receptor did not affect the acute phase of pancreatitis but significantly retarded regeneration of pancreatic tissue [Miyasaka K, Ohta M et al, 1998].

Weight of Evidence Summary

Biological Plausibility

KER	KE_{up}-KE_{down}	Plausibility	Rationale supported by the literature

KER1	Trypsin inhibition increases the luminal concentration of MP	Middle	In rodents, a certain level of monitor peptide (MP) is secreted from pancreatic acinar cells, even between meals or under fasting conditions. However, intestinal MP level is maintained at a low level because of its rapid degradation by trypsin and other proteases (or because of MP–trypsin complex formation, which decreases the level of luminal free MP) [Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998]. After ingestion of trypsin inhibitor (TIs), the intestinal content of MP increases rapidly especially in the fasting state [Iwai K et al, 1988; Liddle RA, 1995]. In other species, trypsin-sensitive CCK-releasing peptide (luminal CCK-releasing factor, LCRF) is released from small intestinal mucosal cells [Owyang C, 1999]. TIs increase the luminal concentration of LCRF; however, the increase in LCRF is not as high compared with MP [Liddle RA, 1995].
KER2	The increased luminal concentration of MP increases the blood CCK level	Strong	In rats, CCK release from I cells in the small intestinal mucosa is regulated by trypsin-sensitive MP [Miyasaka K et al, 1989; Cuber JC et al, 1990; Guan D et al, 1990]. In the empty intestine after dietary protein is digested, secreted MP forms complex with trypsin to be degraded, and luminal level of free MP is kept at low levels, during which CCK release is suppressed Once TIs are ingested, the intestinal concentration of free MP is increased due to trypsin–TI interactions [Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998].Then, the increased MP directly stimulates I cells via their surface MP receptors to release CCK into the blood, leading to increased plasma CCK levels [Bouras EP, 1992; Cuber JC, 1990; Guan D, 1990]. The luminal MP level is further increased along with increased pancreatic exocrine secretion due to the increased plasma level of CCK via a positive feedback loop and trypsin inhibition [Liddle RA, 1995]. In other species including rats, TI increases the luminal level of trypsin-sensitive LCRF to stimulate CCK release, but the increase is transient due to the lack of the positive feedback loop between CCK and LCRF [Liddle RA, 1995].
KER3	The increased blood CCK level stimulates exocrine secretion by pancreatic acinar cells	Strong	Pancreatic exocrine secretion is regulated by CCK released from CCK-producing I cells located in the small intestinal mucosa. CCK stimulates exocrine secretion from pancreatic acinar cells directly via surface CCK receptors and indirectly via vagal afferent nerves expressing CCK receptors in rats. However, in humans, pancreatic secretion is innervated by vagal afferent nerves [Pandiri AR, 2014]. Of the two types of CCK receptors (CCK1 and CCK2 receptors), the former show high affinity to CCK and the latter high affinity to both CCK and gastrin [Dufresne M et al, 2006]. In rats, CCK1 receptors are expressed on pancreatic acinar cells and vagal afferent nerves. On the other hand, in humans, CCK1 receptors are expressed on vagal afferent nerves but not on pancreatic acinar cells, on which CCK2 receptors are expressed instead. CCK2 receptors are not involved in acinar cell functions [Pandiri AR, 2014].

KER4	The exocrine secretion induced by pancreatic acinar cells increases proliferation of pancreatic acinar cells	Strong	An increased plasma level of CCK directly induces proliferation of pancreatic acinar cells via surface CCK1 receptors, as well as exocrine secretion, in rats [Yanatori Y and Fujita T, 1976; Folsch UR et al, 1978; Longnecker DS, 1987; Povoski SP et al, 1994; Tashiro M et al, 2004]. However, the involvement of vagal afferent innervation in acinar cell proliferation under an increased blood level of CCK might be low in humans, but this is unclear [Chandra R and Liddle RA, 2009].
KER5	The increased proliferation of pancreatic acinar cells leads to pancreatic acinar cell tumor formation	Strong	A sustained increase in acinar cell proliferation promotes tumor formation [McGuinness EE et al, 1985]. An increased blood CCK level is the main factor involved in sustained acinar cell proliferation, which promotes acinar cell tumor formation [Douglas BR et al, 1989].

Empirical Support

KER	Empirical support for KERs
MIE=>KE1 Trypsin inhibition increases the luminal concentration of MP	Empirical support for the MIE => KE1 is strong. Rationale No study has demonstrated a direct relationship between trypsin inhibition and an increased luminal concentration of monitor peptide (MP). However, several studies have reported a relationship between trypsin inhibitor (TI) treatment and an increased plasma CCK level. Considering that MP directly stimulates CCK release from I cells in the small intestine in rodents, increased plasma CCK levels induced by TIs suggest increased luminal MP levels. The plasma CCK8 level in rats after 18-hour fasting was 0.31 ± 0.05 pM (mean ± SE) and increased to 6.2 ± 1.8 pM 7.5 minutes after feeding and increased to 10.3 ± 1.8 pM 15 minutes after intragastric instillation of a soybean trypsin inhibitor [Liddle RA et al, 1984]. Immediately after oral feeding of camostat at 400 mg/kg in rats, the plasma CCK level increased 10-fold above that in controls, reached a maximum after 90 min, remained elevated for more than 6 h, and then returned to control levels 24 h after administration of camostat [Goke B et al, 1986].

KE1 =>KE2: An increase in the luminal concentration of MP increases the blood CCK level	Empirical support for the KE1 => KE2 AO is strong. Rationale MP at concentrations ranging from 3 x 10^{-12} to 3 x 10^{-8} M stimulated CCK release from isolated mucosal cells of the rat duodenum in a dose-dependent manner with highest level at 15 minutes after stimulation [Bouras EP et al, 1992]. MP at a concentration range of 2–12 µg/mL induced within a few minutes a dose-dependent transient increase in portal CCK-like immunoreactivity in isolated vascularly perfused rat duodenum/jejunum [Cuber JC et al, 1990]. In rats with biliary and pancreatic fistulas, duodenal infusion of MP at 0.9 µg/rat increased pancreatic secretion and the plasma CCK level [Miyasaka K et al, 1989]. Sorted CCK-positive rat intestinal mucosal cells stimulated with 30 nM MP increased the secretion of CCK in a time-dependent manner as soon as 5 min after the start of stimulation [Liddle RA et al, 1992].
KE2 =>KE3: An increase in the blood CCK level induces exocrine secretion by pancreatic acinar cells	Empirical support of the KE2 => KE3 is strong. Rationale In rats, diversion of bile pancreatic juice induced more than ten-times increase in plasma concentration of CCK at the end of two hours and caused rapid and sustained increase in pancreatic protein secretion with more than two folds at 60 minutes of diversion compared with the basal levels [Li Y and Owyang C, 1994]. Repeated injections of cholecystokinin (CCK) at 130 IU s.c. for 3 weeks significantly increased the pancreatic content and secretion of amylase and trypsin during stimulation with 60 IU/kg-hour of CCK. Peak secretion rates of the enzymes were obtained 45 minutes after the start of the stimulation [Folsch UR et al, 1978]. CCK-mediated feedback control of pancreatic enzyme secretion is also observed in humans. Intraduodenal perfusion of phenylalanine at 10mM, 5mL/min induced a several times increase in the plasma level of CCK within 15 minutes and a four-times increase in one-hour pancreatic outputs of trypsin and chymotrypsin. Simultaneous intraduodenal perfusion of trypsin with phenylalanine lowered plasma CCK level at 24% and pancreatic output of chymotrypsin at 63% compared with the perfusion of phenylalanine alone. Moreover, intravenous infusion of CCK-8 at 20 and 40 ng/kg/h for 60 minutes showed a dose-dependent increase in pancreatic output of chymotrypsin [Owyang C et al, 1986].

KE3 =>KE4: Induction of exocrine secretion by pancreatic acinar cells increases proliferation of pancreatic acinar cells	Empirical support for the KE2 => KE3 is strong. Rationale KE3/KE4: In rats fed 20, 40, and 100% RSF-containing diet for up to 36 weeks, pancreatic hypertrophy was found in all RSF-fed groups, and hyperplasia was found only in the 40 and 100% RSF-fed groups [Crass RA and Morgan RG, 1982]. KE3: Intraduodenal administration of 30 mg RSF increased the total amount of 1-hour pancreatic protein output at 2.2 ± 1.1 mg/h (mean ± SE) in rats in which bile and pancreatic juice were returned to the duodenum [Jordinson M et al, 1996]. KE4: In rats, administration of TIs in drinking water (“Trypsin soybean inhibitor” (Miles), 400mg/100mL) or injection of CCK (CCK-PZ or CCK-33,400 Ivy Dog unit) for 7 days increased acinar cell proliferation as well as acinar cell hypertrophy [Yanatori Y and Fujita T, 1976], and RSF feeding at libitum increased acinar cell proliferation from 7 to 28 days of treatment leading to hypertrophy and hyperplasia [Oates PS and Morgan RG, 1984]. These results show that trypsin inhibition-induced acinar cell proliferation (hyperplasia) developed at higher doses of RSF compared with those of pancreatic hypertrophy caused by increased secretion, or that pancreatic exocrine secretion and increased acinar cell proliferation were detected after 1 h and 7 days, respectively, after the start of TI or CCK treatment.
KE4 =>AO: Increased proliferation of pancreatic acinar cells induces pancreatic acinar cell tumors	Empirical support for the KE4 => AO is strong. Rationale Rats were fed a diet containing 100 or 200 mg TI concentrates prepared from RSF or potato juice. KE4: After 28 days of feeding, both sources of TI induced pancreatic hypertrophy. AO: After 95 weeks of feeding, both TIs induced dose-related pancreatic changes in terms of nodular hyperplasia and acinar adenoma [Gumbmann MR et al, 1989]. Rats continuously fed a diet containing 5% or more RSF developed pancreatic micro/macroscopic nodules and stimulated azaserine-induced nodular hyperplasia and tumorigenesis, and those fed a diet containing 25, 50 and 100% RSF 2 days per week developed pancreatic macro/microscopic nodules, and 100% RSF-fed rats developed pancreatic cancer [McGuinness EE and Wormsley KG, 1986]. Rats fed a diet containing as little as 0.02% camostat 3 days per week developed pancreatic hypertrophy and hyperplasia [Lhoste EF et al, 1988]. F344 rats injected s.c. twice with azaserine at 30 mg/kg BW and treated with camostat at 200 mg/kg BW by gavage 5 days a week for 18 weeks developed azaserine-induced pancreatic preneoplastic lesions. In azaserie-treated Lewis rats, treatment with camostat in diet at 0.5 g/kg diet for 4 weeks and then 0.2 mg/kg diet 3 consecutive days per week for 8 or 16 weeks also promoted the growth of azaserine-induced neoplastic lesions [Lhoste EF et al, 1988].

Quantitative Consideration

KER1:

No study has shown a dose–response relationship between trypsin inhibition and the luminal concentration of MP in rodents. (further research is needed).

KER2:

MP at concentrations ranging from 3×10^{-12} to 3×10^{-8} M stimulated CCK release within 5 minutes from isolated mucosal cells from the rat duodenum in a dose-dependent manner [Bouras EP et al, 1992].

MP at a concentration range of 2–12 µg/mL induced a dose-dependent transient (within several minutes) increase in portal CCK-like immunoreactivity in isolated vascularly perfused rat duodeojejunum. MP at 36 µg/mL showed lower CCK release [Cuber JC et al, 1990].

KER3:

The effect of CCK on the stimulation of pancreatic secretion is dose dependent.

Intravenous infusion of CCK-8 at 20 and 40 pM/kg/hour or high affinity CCKR agonist CCK-JMV-189 at 22, 44 and 88 µg/kg/hour in rats induced dose-dependent increases in pancreatic protein secretion from 15 minutes of infusion [Li Y et al, 1997].

Physiological plasma CCK doses (up to ~10 pM) stimulate the vagal afferent pathway, whereas supraphysiological CCK doses stimulate intrapancreatic neurons and pancreatic acini to secret pancreatic protein [Owyang C, 1996].

KER4:

In rats injected subcutaneously with CCK at 7.5 or 30 Ivy dog units (IU) twice daily for 20 days, pancreatic wet weight and DNA content / 100g BW increased with a same manner compared with saline-treated rats, however, pancreatic output of amylase and trypsin in response to submaximal intravenous stimulation with CCK at 15 IU/kg/hour increased with dose-dependent manner [Folsch UR et al, 1978].

Rats were fed diets consisting of four concentrations of purified soybean TIs (93, 215, 337, and 577 mg/100 g diet) and three protein concentrations (10%, 20%, and 30%) and were then sacrificed at 3-month intervals starting at 6 months [Rackis JJ et al, 1985]. Trypsin and chymotrypsin activities per 100g BW, RNA and DNA contents of pancreas indicative of pancreatic hypertrophy and hyperplasia, respectively, were already increased in all of the TI and protein-fed animals after 6-month dosing, although pancreatic nodules were increased in number at 15 months of dosing or later at 215 mg TI/100 g diet or higher [Liener IE et al, 1985].

KER5:

Rats were fed diets consisting of four concentrations of purified soybean TIs (93, 215, 337, and 577 mg/100 g diet) and three protein concentrations (10%, 20%, and 30%) and were then sacrificed at 3-month intervals starting at 6 months [Rackis JJ et al, 1985]. RNA and DNA contents of pancreas indicative of pancreatic hypertrophy and hyperplasia, respectively, were already increased in all of the TI- and protein-fed animals after 6-month dosing. Pancreatic nodules were increased in number at 15 months of dosing or later and at 215 mg TI/100 g diet or higher[Liener IE et al, 1985].

Considerations for Potential Applications of the AOP (optional)

TBD

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Appendix 1

List of MIEs in this AOP

Event: 1720: Trypsin inhibition (https://aopwiki.org/events/1720)

Short Name: Inhibition, trypsin

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:316 - Trypsin inhibition leading to pancreatic acinar cell tumors (https://aopwiki.org/aops/316)	MolecularInitiatingEvent

Biological Context

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

TBD

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Macaca fascicularis	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Trypsin is a digestive enzyme expressed in many vertebrates, and its molecular weight and isoforms vary among animal species, for example, human cationic and anionic trypsins (trypsins 1 and 2) and mesotrypsin, bovine cationic and anionic trypsins, and rat anionic trypsin and P23 [Chen JM and Claude Férec C, 2013; Fukuoka S and Nyaruhucha CM, 2002]. However, their three-dimensional structures are highly conserved among species [Baird Jr TT, 2013].

The natural substrate for trypsin is generally any peptide that contains Lys or Arg. The active site of trypsin has a specific catalytic triad structure composed of serine, histidine, and aspartate, and the flanking amino acid sequences are entirely conserved [Baird Jr TT and Craik CS, 2013; Baird Jr TT, 2017].

Therefore, TIs show comparable enzymatic inhibition of trypsin molecules among animal species including humans and rats [Savage GP and Morrison SC, 2003].

Key Event Description

Trypsin is a digestive enzyme secreted by pancreatic acinar cells that cleaves peptide bonds at the carboxyl end of basic amino acids (lysine and arginine). Acinar cells secrete trypsinogen, the inactive form of trypsin, into the lumen of the duodenum; in turn, trypsinogen is auto-hydrolyzed by enterokinase into β-trypsin, composed of an uncleaved single chain, and α-trypsin, composed of two cleaved chains bound by a disulfide bridge [Santos AMC et al, 2008]. Trypsin is required for the partial hydrolysis of chymotrypsinogen to chymotrypsin, and most pancreatic digestive enzyme precursors are activated by trypsin in the same manner as chymotrypsin in the intestinal lumen.

As part of the defense against trypsin-induced self injury in the pancreas, internal TIs such as the serine protease inhibitor Kazal type 1 (SPINK1 or human pancreatic trypsin inhibitor) and bovine pancreatic TI in the pancreatic juice and α1-antitrypsin in the serum bind tightly to active trypsin [Voet D and Voet JG, 1995].

Secretion of pancreatic digestive enzymes including trypsin is regulated mainly by CCK released from enteroendocrine I cells in the duodenal mucosa of the small intestine [Wang BJ and Cui ZJ, 2007], and CCK release is controlled by multiple mechanisms [Caron J et al, 2017]. One such mechanism is trypsin-mediated negative feedback regulation, in which increased trypsin secretion leads to decreased levels of trypsin-sensitive luminal CCK-releasing factors (LCRFs) in several mammalian species and MP in rodents [Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998].

Therefore, ingestion of RSF containing trypsin inhibitory action or protease inhibitors such as camostat inhibits trypsin activity in the intestinal lumen, which leads to increased luminal levels of the abovementioned trypsin-sensitive peptides and thereby stimulation of CCK release [Green GM and Miyasaka K, 1983; Cuber JC et al, 1990; Miyasaka K et al, 1989; Cuber JC et al, 1990; Komarnytsky S et al, 2011].

How it is Measured or Detected

Activity of trypsin inhibitors is measured colorimetrically using mixture of multiple dilutions of samples (TIs), trypsin and its substrate. Standard procedures for measuring TI activities in soy bean products are released as AACCI Method 22-40.01 [AACCI, 2009] and AOCS Method Ba 12-75 [AOCC, 2017]. ISO standard for measuring TI activities is also established as Standard 14902:2001 [ISO, 2012]. The two methods of modified AACC 20-40.01 and ISO 14902 were compared to show that the values obtained by these two methods are not directly comparable [Sueiro S et al, 2015]. Modified standard method is proposed reconsidering the levels of dilutions and volumes, reaction sequence and other factors [Liu K, 2019].

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List of Key Events in the AOP

Event: 1721: Increased intestinal monitor peptide level (https://aopwiki.org/events/1721)

Short Name: Increased monitor peptide

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:316 - Trypsin inhibition leading to pancreatic acinar cell tumors (https://aopwiki.org/aops/316)	KeyEvent

Biological Context

Level of Biological Organization
Organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Macaca fascicularis	Macaca fascicularis	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Feedback regulation of pancreatic enzyme secretion mediated by trypsin-sensitive intestinal peptides other than MP has been reported in mammals. Such peptides include luminal CCK-releasing factors (LCRFs) secreted by duodenal mucosal cells in response to intestinal diet in some mammalian species including rats, pigs (diazepam-binding inhibitor) and humans [Miyasaka K and Funakoshi A, 1998; Wang Y, 2002; Wang BJ and Cui ZJ, 2007]. In humans, different from rodents, LCRF is not secreted spontaneously in the intestine, however luminal amino acids and fatty acids were reported to induce CCK release [Liddle RA, 1997].

MP is one of pancreatic soluble TIs (PSTIs), which are found in the pancreatic juice of many mammalian species including pigs, dogs, and humans [Greene LJ et al, 1968; Pubols MH et al, 1974; Eddeland A and Ohlsson K, 1976; Kikuchi N et al, 1985]. Secreted PSTIs bind tightly to trypsin to protect against trypsin-induced auto-injury in the pancreas and intestinal tracts [Voet D and Voet JG, 1995].

In rats, two types of PSTIs have been isolated: MP (or PSTI-I) and PSTI-II [Tsuzuki S et al, 1991; Tsuzuki S et al, 1992]. Both are similar in amino acid sequence; however, the former directly stimulates CCK release from intestinal CCK I cells via their surface MP receptors [Yamanishi R et al, 1993], whereas the latter does not [Guan D et al, 1990].

Human PSTIs do not directly stimulate CCK release from intestinal mucosal cells [Miyasaka K et al, 1989], and no PSTI except MP has been reported to stimulate CCK release.

Key Event Description

Trypsin-mediated feedback regulation of pancreatic exocrine secretion is commonly found among vertebrate species.

In rats, trypsin-sensitive monitor peptide (MP), a pancreatic soluble trypsin inhibitor (TI) found in pancreatic juice that protects against trypsin-induced auto-injury [Iwai K et al, 1987; Iwai K et al, 1988; Tsuzuki S et al, 1991; Tsuzuki S et al, 1992], plays a major role in stimulating pancreatic exocrine secretion via cholecystokinin (CCK) release [Miyasaka K et al, 1989; Fushiki T et al, 1989; Miyasaka K and Funakoshi A, 1998].

MP is a peptide consisting of 61 amino acids with a molecular weight of approximately 6000 and is secreted from pancreatic acinar cells along with other pancreatic enzymes [Iwai K et al, 1987].MP is reported to have trypsin inhibitory activity [Lin YZ et al, 1990], and it forms complexes with trypsin in the empty intestine, similar to other pancreatic soluble TIs [Voet D and Voet JG, 1995], which keeps the intestinal level of free MP low. However, once the gastric contents are transported to the small intestine, secretion of the pancreatic proteases with MP are induced, where trypsin is used for protein hydrolysis, and the level of free MP is subsequently increased [Iwai K et al, 1988; Graf R, 2006]. The increased MP level stimulates CCK release from I cells lining the small intestinal mucosa via MP receptors [Liddle RA et al, 1992; Yamanishi R et al, 1993; Yamanishi R et al, 1993; Liddle RA et al, 1992], and the resulting increase in CCK stimulates exocrine secretion from the pancreas. MP secretion is simultaneously increased to stimulate CCK release further. Therefore, MP-mediated regulation of trypsin and related proteases appears to act via a positive feedback loop as long as duodenal contents remain to consume trypsin for proteolysis.

In accordance with the increased secretion of pancreatic enzymes, proteolysis of the intestinal contents lowers protein levels in the intestinal lumen, which once again lowers the intestinal level of free MP due to the excess of trypsin. CCK release is decreased in accordance with the decreased intestinal MP level, followed by a decrease in pancreatic exocrine secretion [Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998].

After ingestion of raw soya flour, which contains trypsin inhibitory activity, or TIs such as camostat, TI–trypsin complexes are formed, and the intestinal level of free MP is increased to stimulate CCK release [Yamanishi R et al, 1993], increasing the blood CCK level. Increased CCK further stimulates MP as well as other pancreatic enzymes via positive feedback regulation [Liddle RA, 1995].

How it is Measured or Detected

No literatures that describe the methods of measuring intestinal concentration of MP are found although some authors reported the isolation and measurement of MP from synthesis reaction solution, pancreas or pancreatic juice.

Synthesized crude peptides were eluted through gel filtration chromatography. PSTI-I-specific peak was confirmed by mas spectrometric measurement and analytical HPLC was performed [Graf R et al, 2003].

In rats fed control and high protein diets, zymogen granules were isolated and concentrations of MP and PSTI-II in zymogen granules can be determined by HPLC [Tsuzuki S et al, 1991].

Rat anionic trypsinogen and a pancreatic secretory trypsin inhibitor were purified from the pure pancreatic juice of rats by reverse-phase HPLC [Iwai K et al, 1987].

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Event: 1722: Increased blood CCK level (https://aopwiki.org/events/1722)

Short Name: Increased blood CCK level

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:316 - Trypsin inhibition leading to pancreatic acinar cell tumors (https://aopwiki.org/aops/316)	KeyEvent

Biological Context

Level of Biological Organization
Organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Macaca fascicularis	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

There are species differences in the regulation of CCK release.

Fats, fatty acids, proteins, and amino acids stimulate CCK release in humans, and fatty acids and amino acids are the key factors regulating CCK release in dogs. These factors stimulate intestinal I cells to release CCK either directly via cell surface receptors such as Ca-sensing receptors and the G protein-coupled receptor GPR93 or indirectly via LCRFs [Caron J et al, 2017]. Amino acids directly stimulate LCRF release from small intestinal mucosal cells in humans [Wang BJ and Cui ZJ, 2007].

On the other hand, in rodents, trypsin-mediated negative and positive feedback regulation loops involved in CCK release have been identified; the former is mediated by LCRF secreted from intestinal mucosal cells and the latter via MP secreted from pancreatic acinar cells [Liddle RA, 1995; Wang BJ and Cui ZJ, 2007; Miyasaka K and Funakoshi A, 1998]. This mechanism of CCK release regulation is plausible in rodents, because of their diet of wild legumes and cereal grains, which contain trypsin inhibitors, and the short digestion time in the stomach.

Multiple isoforms of CCKs (e.g., CCK-83, -58, -39, -33, -22, -8, and others) have been identified, and their expression differs among species (humans express CCK-33, -22, and -58; dogs express CCK-58; cats express CCK-8, -33, and -58; pigs express CCK-22, -58, -3, and -8; rabbits express CCK-22 and -8; and rats express CCK-58). All CCK isoforms contain a highly conserved region of amino acids and serve as ligands for CCK1 receptors [Wang BJ and Cui ZJ, 2007; Rehfeld JF, 2017].

Key Event Description

Pancreatic exocrine secretion is controlled by multiple mechanisms [Caron J et al, 2017; Wang BJ and Cui ZJ, 2007; Wang Y et al, 2011], many of which are mediated by CCK secreted by CCK-producing I cells lining the mucosa of the small intestine [Singer MV and Niebergall-Roth E, 2009; Rehfeld JF, 2017]. CCK is also synthesized in cerebral neurons and expressed in several endocrine and certain other cells, and they are involved in many functions other than pancreatic exocrine secretion, including gall bladder contraction, gut motility, and satiety [Rehfeld JF, 2017].

CCK is initially synthesized as a peptide prohormone comprising 150 amino acids, which is processed into active CCK by prohormone convertases specific to the cell type and species [Rehfeld JF et al, 2003; Wang BJ and Cui ZJ, 2007]. CCKs exist as several isoforms that differ due to post-translational modifications, although the C-terminal amino acid sequences are conserved among these isoforms [Rehfeld JF et al, 2001; Rehfeld JF, 2017].

CCK release is stimulated mainly by gastric contents containing fatty acids and amino acids transported into the small intestine. The factors in gastric chyme that stimulate CCK release differ among species, with fats, fatty acids, proteins, and amino acids being the key players in humans, fatty acids and amino acids in canines, and digested/undigested proteins in rats [Wang BJ and Cui ZJ, 2007; Caron J et al, 2017]. These factors stimulate intestinal mucosal I cells to release CCK into the blood either directly via specific receptors such as calcium-sensing receptors and the G protein-coupled receptor GPR93 or indirectly via luminal CCK-releasing factors (LCRFs) [Caron J et al, 2017]. LCRFs are released from intestinal mucosal cells in response to amino acids and fatty acids in humans [Liddle RA, 1997; Liddle RA, 2000] ; however, the peptides mediate negative feedback regulation of CCK release via CCK degradation by pancreatic proteases [Wang BJ and Cui ZJ, 2007].

In addition to the negative feedback regulation of CCK release in rodents, CCK release is stimulated by monitor peptide (MP), a pancreatic soluble trypsin inhibitor (PSTI) secreted into the upper intestine from pancreatic acinar cells [Wang BJ and Cui ZJ, 2007]. MP, which is trypsin-sensitive, stimulates intestinal I cells to release CCK via positive feedback regulation, in that the resulting increased CCK level stimulates the secretion of MP together with other pancreatic enzymes [Liddle RA, 1995; Wang BJ and Cui ZJ, 2007; Miyasaka K and Funakoshi A, 1998]

When trypsin is inhibited in rodents, trypsin-sensitive MP-induced CCK release is overstimulated due to positive feedback regulation of CCK release by trypsin.

How it is Measured or Detected

Plasma was first extracted on octadecylsilyl silica columns, and the CCK concentration was measured in the resulting extracts, based on the ability of the extracts to stimulate amylase release from isolated rat pancreatic acini [Liddle RA et al, 1984].

The STC-1 cell line, which is derived from murine enteroendocrine tumor cells, secretes several enteroendocrine hormones including CCK, GLP-1, and GLP-2 in response to many different stimulants such as monosaccharides, aromatic amino acids, peptidomimetic compounds, and bitter tastants [Wang BJ and Cui ZJ, 2007].

CCK release from STC-1 cells or intestinal cell preparation were measured by sensitive and specific radioimmunoassay, which recognizes biologically active forms of CCK [Wang Y et al, 2002; Wang Y et al, 2011].

In order to assess the effects of protein hydrolysates on CCK release from enteroendocrine cells, each of protein hydrolysates and STC-1 cells were incubated and CCK release is measured by ELISA [Foltz M et al, 2008].

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Event: 1723: Increased exocrine secretion from pancreatic acinar cells (https://aopwiki.org/events/1723)

Short Name: Increased acinar cell exocrine secretion

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:316 - Trypsin inhibition leading to pancreatic acinar cell tumors (https://aopwiki.org/aops/316)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Macaca fascicularis	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

CCK1 and CCK2 receptors are expressed in various organs and tissues including the digestive and nervous systems, and there are species differences in the expression localization and levels of these receptors.

In rats, it has been reported that pancreatic acinar cells express mainly CCK1 receptors but not CCK2 receptors [Bourassa J et al, 1999]. CCK1 receptors are also expressed in vagal afferent nerve fibers of the gastroduodenal tract; stimulation of the vagus nerve via CCK1 receptors as well as via physical stimulation of stomach wall distention by ingested food also promotes pancreatic exocrine secretion [Dufresne M et al, 2006].

Meanwhile, in humans, CCK2 receptors are dominantly expressed in pancreatic acinar cells, with low expression of CCK1 receptors [Nishimori I et al, 1999]. Ji reported the following using human pancreatic acini: 1) the mRNA level of the CCK2 receptor is higher than that of the CCK1 receptor,

2) an in situ hybridization experiment showed no expression of either receptor type, and 3) human pancreatic cells did not show any response to the CCK1 receptor agonist CCK8 or the CCK2 receptor agonist gastrin in vitro [Ji B et al, 2001]. Therefore, human pancreatic acinar cells respond to CCK more weakly compared with rodents, because the CCK receptor subtypes expressed in the pancreas are different between humans and rodents, and CCK receptor expression levels are lower in humans than rodents.

In addition, exocrine secretion from the human pancreas is regulated mainly by innervation of vagal afferent nerves via CCK1 receptors and less so by direct stimulation of acinar cells via CCK receptors [Wang BJ and Cui ZJ, 2007; Owyang C, 1996; Pandiri AR, 2014]. Although the distribution of CCK receptors is different between humans and rodents, the structures of CCK1 receptors are highly conserved among mammalian species, with 98% homology between rats and mice, 90% between rats and humans, 98% between cynomolgus monkeys and humans, and 89% between dogs and humans [Wang BJ and Cui ZJ, 2007].

Multiple molecular forms of CCKs, including CCK-83, -58, -39, -33, -22, -8 among others, have been identified; all of these isoforms have a highly conserved region of 5 amino acid sequences at the C-terminal, and all are ligands for CCK1 receptors [Wank SA, 1995].

Key Event Description

The major function of pancreatic exocrine secretion is secretion of digestive enzymes, fluid, and bicarbonate in response to food intake. Zymogen granules located at the apical site of pancreatic acinar cells contain the precursors of multiple digestive enzymes, such as trypsinogen, chymotrypsinogen, proesterase, procarboxypeptidase A and B, as well as pancreatic lipase and amylase α. These precursors are secreted into the small intestine, where trypsinogen is converted to trypsin by enteropeptidase, and the newly generated trypsin activates more trypsinogen molecules and other proenzymes [Berg JM et al, 2002].

Pancreatic exocrine secretion is regulated mainly by CCK released from CCK-producing I cells located within the mucosa of the small intestine. CCK stimulates exocrine secretion either directly via CCK receptors expressed on acinar cells or indirectly by the vagovagal reflex via CCK receptors. There are species differences in these CCK regulatory mechanisms [Singer MV and Niebergall-Roth E, 2009; Chandra R and Liddle RA, 2009].

There are two types of CCK receptors: CCK1 (CCK-A) and CCK2 (CCK-B or gastrin) receptors. The CCK1 receptor exhibits high affinity to all CCK isoforms, whereas the CCK2 receptor exhibits affinity to both CCK and gastrin, in which the last five amino acid sequences at the C-terminus end are identical [Dufresne M et al, 2006; Rehfeld JF, 2017].

In rats, pancreatic acinar cells express mainly CCK1 receptors, and blood CCK directly stimulates exocrine secretion and acinar cell proliferation [Dufresne M et al, 2006]. Moreover, the vagal afferent nerves also stimulate pancreatic exocrine secretion; CCK stimulates CCK1 receptors expressed on the vagal afferent nerve fibers of the vago–vagal reflex loop, and the acetylcholine generated acts on M3 muscarinic cholinergic receptors to promote pancreatic exocrine secretion [Bourassa J et al, 1999; Adler G, 1997; Ji B et al, 2001; Li Y et al, 1997; Owyang C, 1996]. Moreover, when the gastric wall is distended with ingested food, the vagus nerve is stimulated to promote pancreatic exocrine secretion [Dufresne M et al, 2006].

In humans, the density of CCK receptors expressed on acinar cells is lower than that in rodents, whereas CCK2 receptors are dominantly expressed. Therefore, the responses of acinar cells to CCK seem to be weaker compared with rodents, and pancreatic exocrine secretion in humans is regulated mainly by vagal afferent nerves expressing CCK1 receptors [Wang BJ and Cui ZJ, 2007; Owyang C, 1996; Pandiri AR, 2014].

How it is Measured or Detected

Ex vivo procedure for measuring secretion from pancreatic acini is reported [Geron E, 2014], where ex vivo culture of pancreatic acini isolated from mice is used for amylase secretion assay as a global measure and direct imaging of pancreatic secretion with subcellular resolution.

The release of amylase was measured in dispersed acini from human pancreas [Miyasaka K et al, 2002].

Pancreatic exocrine secretion was measured in rats with chronic pancreatitis and pancreatic and biliary fistulas [Green GM and Miyasaka K, 1983]. Pancreatic juice was collected from the jejunum and the amounts of protein and pancreatic enzymes were measured.

Pancreatic enzyme activities in pancreatic outlet were measured after CCA injection [Folsch UR et al, 1978]. After repeated subcutaneous injections of CCK in rats, pancreatic enzymes were collected by perfusing duodenum. trypsin in the perfusate was activated with enterokinase and its activity was measured photometrically using benzoyl arginine as substrate. Amylase activity in the perfusate was measured using Zulkovsky starch as substrate. The concentration of protein per weight of DNA, the total level of pancreatic DNA, and the pancreatic levels of amylase and trypsin were also measured in rats after repeated CCK administration.

In the rats fed the TI camostat, pancreatic weight and protein, DNA, and enzyme contents and trypsinogen, chymotrypsinogen, and amylase levels in the pancreatic homogenates prepared 24 hours after the last administration were measured [Goke B et al, 1986]. Levels of trypsinogen and chymotrypsinogen were measured as trypsin and chymotrypsin after activation.

References

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9. Green GM, Miyasaka K: Rat pancreatic response to intestinal infusion of intact and hydrolyzed protein. Am J Physiol 245:G394-8,1983

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17. Singer MV, Niebergall-Roth E: Secretion from acinar cells of the exocrine pancreas: role of enteropancreatic reflexes and cholecystokinin. Cell Biol Int 33:1-9,2009

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19. Wank SA: Cholecystokinin receptors. Am J Physiol 269:G628-646,1995

Event: 1724: Acinar cell proliferation (https://aopwiki.org/events/1724)

Short Name: Acinar cell proliferation

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:316 - Trypsin inhibition leading to pancreatic acinar cell tumors (https://aopwiki.org/aops/316)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
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Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

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List of Adverse Outcomes in this AOP

Event: 1725: Pancreatic acinar cell tumors (https://aopwiki.org/events/1725)

Short Name: Acinar cell tumors

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:316 - Trypsin inhibition leading to pancreatic acinar cell tumors (https://aopwiki.org/aops/316)	AdverseOutcome

Biological Context

Level of Biological Organization
Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Macaca fascicularis	Macaca fascicularis	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Mus musculus	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

In monkeys receiving repeated dosing of the CCK1 receptor agonist GI181771X for up to 52 weeks, no hypertrophy or histopathological changes of the pancreas were observed, but these results differed from those of rats [Myer JR et al, 2014]. In humans, obese patients treated with GI181771X for 24 weeks showed no abnormal changes in the pancreas by ultrasonography or MRI [Jordan J et al, 2008]. On the other hand, oral ingestion of raw soya flour, which contains trypsin inhibitors, increased the release of CCK in humans [Calam J et al, 1987]. In addition, some epidemiological studies reported that the incidence of pancreatic tumors in humans administered high levels of protease inhibitors was decreased [Messina M and Messina V, 1991; Miller RV, 1978]. These results suggest no relevance between pancreatic growth/tumor development and CCK-agonist treatment in humans or non-human primates.

As indicated above, the effects of CCK on acinar cell proliferation differ between rodents and humans. In rodents, proliferation of pancreatic acinar cells is regulated directly via CCK1 receptors expressed on their surfaces. However, in humans, CCK1 receptor density on the surface of pancreatic acinar cells is low, and exocrine secretion is innervated by vagal afferent nerves, with little effect on acinar cell proliferation.

Key Event Description

Several reports have shown that increased blood CCK levels directly stimulate acinar cell proliferation via CCK1 receptors in rats as follows:

In rats with a sustained increase in the CCK level due to treatment with a CCK1 receptor agonist (CCK-8), acinar cell proliferation and pancreatic hypertrophy were induced [Folsch UR et al, 1978; Povoski SP et al, 1994]. Endogenous and exogenous increases in blood CCK levels induced pancreatic hypertrophy due to the direct action of CCK on acinar cells [Yamamoto M et al, 2003]. Repeated administration of the CCK1 receptor agonist GI181771X to rats and mice resulted in pancreatic injury, hypertrophy and diffuse/focal hyperplasia of acinar cells, and zymogen degranulation depending on the dose and dosing period [Myer JR et al, 2014].

Administration of the trypsin inhibitor A8947 to rats increased pancreatic weight; however, infusion of the selective CCK1 receptor antagonist MK-329 using an osmotic minipump completely diminished this effect of A8947 on pancreatic weight [Obourn JD et al, 1997].

These results indicate that CCK directly stimulates pancreatic acinar cell proliferation via CCK1 receptors, and trypsin inhibition enhances acinar cell proliferation due to an increased plasma level of CCK.

How it is Measured or Detected

Pancreatic acinar cell proliferation is evaluated based on measurements of pancreatic weight and DNA and RNA levels [Folsch UR et al, 1978; Povoski SP et al, 1994; Tashiro M et al, 2004], as well as histopathological examination [Povoski SP et al, 1994]. In these experiment, pancreatic

weight, protein content, RNA content, DNA content, protein-DNA ratio, RNA-DNA ratio, pancreatic area per nucleus, and number of mitoses per 10,000 acinar cells could be determined. Among such parameters, Increased DNA content and number of mitoses per 10,000 acinar cells are indicative of acinar cell hyperplasia, and the others suggest pancreatic or acinar cell hypertrophy and increased pancreatic protein synthesis.

References

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2028: Inhibition, trypsin leads to Increased monitor peptide (https://aopwiki.org/relationships/2028)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Trypsin inhibition leading to pancreatic acinar cell tumors (https://aopwiki.org/aops/316)	**adjacent**	**Moderate**	**Low**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Macaca fascicularis	Macaca fascicularis	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Isoforms of trypsin are found in many species, for example, cationic and anionic trypsins (trypsins 1 and 2) and mesotrypsin in humans, cationic and anionic trypsins in cows, and anionic trypsin and P23 in rats [Chen JM and Claude Férec C, 2013; Fukuoka S and Nyaruhucha CM, 2002] . Despite differences among species, the three-dimensional structures of the isoforms are highly conserved among species, and the natural substrates for the enzymes are generally any peptide that contains Lys or Arg [Baird Jr TT, 2017]. The active site of trypsin has a specific catalytic triad structure composed of serine, histidine, and aspartate, and the flanking amino acid sequences are entirely conserved [Baird Jr TT and Craik CS, 2013; Baird Jr TT, 2017]. Therefore, trypsin inhibitors have comparable effects on the enzymatic activity of trypsin isoforms among animal species including humans and rats [Savage GP and Morrison SC, 2003].

MP secreted from rat pancreatic acinar cells into the small intestine stimulates I cells of the small intestinal mucosa to release CCK.

MP-like peptides are also found in rats and other mammalian species [Eddeland A and Ohlsson K, 1976]. Rat soluble trypsin inhibitor [Tsuzuki S et al, 1992; Tsuzuki S et al, 1991], human soluble trypsin inhibitor [Pubols MH et al, 1974; Kikuchi N et al, 1985], and bovine soluble trypsin inhibitor [Greene LJ and Giordano JS Jr, 1969; Guy O et al, 1971] are homologous peptides, all of which show trypsin inhibitory activity but no CCK-stimulatory activity [Miyasaka K et al, 1989a; Miyasaka K et al, 1989b; Marchbank T et al, 1998; Voet D and Voet JG, 1995].

Key Event Relationship Description

Pancreatic acinar cells secrete digestive enzymes including trypsin into the small intestine.

In rats, one of the pancreatic soluble trypsin inhibitors (TIs), monitor peptide (MP), is simultaneously secreted in the pancreatic juice. MP forms complexes with trypsin in the empty intestine, which keeps the intestinal level of free MP low. Once the gastric contents are transported to the small intestine, secretion of the pancreatic proteases including trypsin and MP is induced, where trypsin is used for protein hydrolysis, and the level of free MP is subsequently increased. The increased MP level stimulates CCK release from I cells lining the small intestinal mucosa via MP receptors, and the resulting increase in CCK stimulates exocrine secretion including MP from the pancreas. Increased MP further stimulates CCK secretion via a positive feedback loop as long as duodenal contents remain to consume trypsin for proteolysis.

After trypsin inhibitors are ingested, the intestinal content of free MP increases rapidly, especially in an empty intestine, via positive feedback regulation.

Evidence Supporting this KER

TBD

Biological Plausibility

Trypsin is a digestive enzyme secreted by pancreatic acinar cells that cleaves peptide bonds at the carboxyl end of basic amino acids (lysine and arginine). Secretion of pancreatic digestive enzymes including trypsin is regulated mainly by cholecystokinin (CCK) released from enteroendocrine I cells located in the duodenal mucosa of the small intestine [Wang BJ and Cui ZJ, 2007], and CCK release is controlled by multiple mechanisms [Caron J et al, 2017]. These mechanisms involve feedback regulation of trypsin-sensitive CCK-releasing peptides, one being positive feedback regulation of MP and the other negative feedback regulation of luminal CCK-releasing factor (LCRF) [Miyasaka K and Funakoshi A, 1998; Wang BJ and Cui ZJ, 2007; Guan D et al, 1990].

MP is one of the PSTIs in rats, which stimulates CCK release from duodenal enteroendocrine I cells as well as inhibition of trypsin activity. MP consists of 61 amino acids and has a molecular weight of approximately 6000. MP was first purified from rat pancreatic juice, and its amino acid sequence was subsequently determined [Iwai K et al, 1987; Lin YZ et al, 1990].

MP is bound to trypsin in the empty intestine. Once gastric contents are transported into the small intestine, secretion of the pancreatic proteases with MP is increased, where trypsin instead hydrolyzes these proteins, leading to an increase in the free MP level [Iwai K et al, 1988; Liddle RA, 1995; Graf R, 2006]. The increased level of MP stimulates CCK release from I cells, and then pancreatic exocrine secretion is stimulated [Liddle RA et al, 1992; Guan D et al, 1990; Cuber JC et al, 1990]. It was shown that MP binds to the surface of CCK-immunoreactive mucosal cells of the small intestine [Yamanishi R et al, 1993a; Yamanishi R et al, 1993b].

Following the increased secretion of pancreatic enzymes, proteolysis decreases intestinal protein contents, which once again decreases the intestinal level of free MP due to the excess of trypsin and in turn CCK release [Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998; Graf R, 2006].

When raw soya flour (RSF), which contains trypsin inhibitory activity, or TIs such as camostat are ingested, trypsin activity is inhibited to increase the intestinal level of free MP especially in the empty intestine, followed by an increase in the blood level of CCK [Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998]. TI ingestion-induced increases in blood levels of CCK leads to further CCK release due to increased pancreatic secretion of proteins including MP in a positive feedback manner. On the other hand, TIs may elevate the luminal concentration of LCRF to stimulate CCK release; however, this increase might not be as exaggerated as that of MP, because increased blood level of CCK does not induce further secretion of LCRF.

Empirical Evidence

No study has shown a relationship between the trypsin inhibitor dose or degree of trypsin activity and the luminal concentration of MP. Trypsin inhibitor dosing and CCK levels are presented here. Considering that MP directly stimulates CCK release from I cells in the small intestine in rodents, increased plasma CCK levels induced by TIs seems to be appropriate as a surrogate of increased luminal MP levels.

The plasma CCK8 level in rats after 18-hour fasting was 0.31 ± 0.05 pM (mean ± SE) and increased to 6.2 ± 1.8 pM 7.5 minutes after feeding and increased to 10.3 ± 1.8 pM 15 minutes after intragastric instillation of a soybean trypsin inhibitor [Liddle RA et al, 1984].

Immediately after oral feeding of camostat at 400 mg/kg in rats, the plasma CCK level increased 10-fold above that in controls, reached a maximum after 90 min, remained elevated for more than 6 h, and then returned to control levels 24 h after administration of camostat [Goke B et al, 1986].

Plasma concentrations of CCK were measured after administration of a single dose of 200 mg/kg camostat by gavage or 2.5 μg/kg CCK8 by subcutaneous administration to rats. The maximum CCK level, 9.6 ± 2.7 pM, was reached at 30 min after administration of CCK8, and that of 4.9 ± 1.2 pM over the time period of 15–240 min per animal with basal CCK concentration of about 2.5 pM [Douglas BR et al, 1989].

In isolated vascularly perfused rat duodenum/jejunum 30-min of infusion of trypsin with ovalbumin hydrolysate reduced CCK release by approximately 60% of that induced by the peptone alone. This effect was reversed by co-infusion of soybean trypsin inhibitor with the trypsin–peptone mixture [Cuber JC et al, 1990].

Eleven healthy volunteers consumed one of two meals: one containing raw soya flour and the other heat-treated soya flour. The two flours contained 34 and 3 mg trypsin/g flour, respectively. The peak CCK response was 16.8 ± 8.1 (mean ± SE) pmol/l for the raw soya flour diet versus 4.9 ± 2.8 pmol/l for the heat-treated soya flour diet ($P < 0.05$) [Calam J et al, 1987].

Uncertainties and Inconsistencies
In normal rats, positive regulation of CCK release by MP seems to require some level of pancreatic secretion before to be effective. In the presence of nutritional protein in the duodenum, trypsin is used for digestion of protein and increased levels of MP stimulates CCK release. On the other hand, after most of the protein is digested, increased free MP might be inactivated with excess of trypsin or other proteases, as follows [Foltz M, 2008]:

1) MP is degraded by trypsin and other proteases.

2) MP forms a complex with trypsin as other PSTIs.

3) MP forms a complex with trypsin, thereafter degraded by proteases.

Quantitative Understanding of the Linkage

TBD

Response-response relationship
No study has shown a direct quantitative relationship between MIE and KE1.

Time-scale
No study has reported the time from trypsin inhibition to alteration of intestinal MP content. However, as mentioned above, treatment with trypsin inhibitors or MP increased the plasma concentration of CCK within 30 min in rats.

Known modulating factors
Raw soya flour and trypsin inhibitors such as camostat inhibit trypsin activity, leading to an increase in CCK release from the upper intestine into the bloodstream, where the increased CCK released seems to be mediated by increased luminal concentration of MP due to trypsin inhibition [Green GM and Miyasaka K, 1983; Liddle RA et al, 1984; Goke B et al, 1986; Douglas BR et al, 1989; Cuber JC et al, 1990; Playford RJ et al, 1993; Obourn JD et al, 1997; Tashiro M et al, 2004; Komarnytsky S et al, 2011; Calam J et al, 1987] .

Known Feedforward/Feedback loops influencing this KER
MP stimulates CCK release from intestinal I cells, and the increased CCK level in turn promotes pancreatic acinar cells to secrete pancreatic enzymes including CCK-stimulating MP. Therefore, MP-mediated CCK release is under positive feedback regulation [Liddle RA, 1995; Wang BJ and Cui ZJ, 2007; Chey WY and Chang T, 2001], and the effects of trypsin inhibitors seem robust. As discussed previously, trypsin-sensitive LCRF released from intestinal mucosal cells also stimulate duodenal I cells to release CCK with negative feedback loop.

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Relationship: 2029: Increased monitor peptide leads to Increased blood CCK level (https://aopwiki.org/relationships/2029)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Trypsin inhibition leading to pancreatic acinar cell tumors (https://aopwiki.org/aops/316)	**adjacent**	**High**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Macaca fascicularis	Macaca fascicularis	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Monitor peptide and related peptides with trypsin inhibitory activity

Pancreatic secretory trypsin inhibitors (PSTIs) are found in the pancreatic juice of multiple mammalian species, including rodents and humans [Greene LJ et al, 1968; Pubols MH et al, 1974; Eddeland A and Ohlsson K, 1976; Kikuchi N et al, 1985]. Secreted PSTIs bind tightly to trypsin to protect against trypsin-induced auto-injury in the pancreas and intestinal tracts [Voet D and Voet JG, 1995].

In rats, two types of PSTIs have been isolated: monitor peptide (MP, also known as PSTI-I) and PSTI-II [Tsuzuki S et al, 1991; Tsuzuki S et al, 1992]. Both are similar in amino acid sequence; however, the former directly stimulates CCK release from intestinal I cells via their surface MP receptors, whereas the latter does not [Miyasaka K et al, 1989b; Yamanishi R et al, 1993a]. Human PSTIs do not directly stimulate CCK release from intestinal mucosal cells [Miyasaka K et al, 1989a]. PSTIs from other mammalian species including dogs and pigs might neither directly stimulate CCK release although no related reports are found.

Species differences in the mechanism of CCK release

Pancreatic exocrine secretion is controlled mainly by CCK released into the bloodstream from intestinal mucosal I cells of the small intestine in response to the gastric contents transported to the intestine [Singer MV and Niebergall-Roth E, 2009; Rehfeld JF, 2017]. Peptides released from gastrointestinal digestion, along with fatty acids, are the main stimuli of CCK release involving several direct and indirect pathways [Caron J et al, 2017].

In humans and canines, amino acids and fatty acids in the gastric contents transported to the small intestine play a major role in stimulating CCK release, which regulates pancreatic exocrine secretion, but MP is not involved in exocrine regulation [Wang BJ and Cui ZJ, 2007].

In rats, in contrast to other mammalian species, MP secreted by pancreatic acinar cells plays a major role in protein-stimulated CCK release [Iwai K et al, 1988; Fushiki T et al, 1989]. Ingestion of TIs increases the intestinal level of MP, especially after all nutrient protein is digested in the intestines, causing a subsequent increase in the blood level of CCK. Increased levels of CCK stimulate pancreatic exocrine secretion of proteins including MP, which in turn further increases the release of CCK. This positive feedback response associated with MP secretion might lead to continuously elevated plasma levels of CCK [Liddle RA, 1995].

Species differences in CCKs

Several isoforms of CCK, including CCK-83, -58, -39, -33, -22, and -8, have been identified, and there are species differences in CCK isoforms (e.g., CCK-33, -22 and -58 are expressed in humans, CCK-58 in dogs, CCK-8, -33 and -58 in cats, CCK-22, -58, -3 and -8 in pigs, CCK-22 and -8 in rabbits, and CCK-58 in rats). All of these CCK isoforms have a highly conserved region of amino acids, and all are ligands of CCK1 receptors [Wang BJ and Cui ZJ, 2007].

Key Event Relationship Description

Pancreatic exocrine secretion is regulated mainly by cholecystokinin (CCK) via multiple mechanisms. In the digestive system, CCK is released by I cells located in the duodenal mucosa of the small intestine. CCK release is at least in part under negative or positive feedback regulation mediated by trypsin-sensitive CCK-releasing peptides.

In rats, CCK release from I cells is regulated actively by monitor peptide (MP) secreted from pancreatic acinar cells in the presence of nutritional protein in the duodenum [Graf R, 2006].

In the empty intestine, secreted MP binds to trypsin and thus maintained at low intestinal levels; in this situation, CCK release is suppressed. Once the gastric contents are transported to the small intestine, secretion of pancreatic juice including trypsin and MP is stimulated, where trypsin is used for digestion, and the level of free MP is subsequently increased. The increased free MP level stimulates CCK release from I cells via MP receptors, and the resulting increase in CCK stimulates pancreatic exocrine secretion including MP. The resulting increased level of MP directly stimulates I cells to release CCK further; this positive feedback regulation might be continued as long as duodenal contents remain to consume trypsin for proteolysis.

Meanwhile, soon after nutritional protein is digested, free MP and excessive trypsin binds together to be subsequently degraded followed by decreases in blood level of CCK and pancreatic secretion. However, after ingestion of trypsin inhibitors, the intestinal concentration of MP is increased continuously with positive feedback manner due to inhibition of its degradation by trypsin.

On the other hand, in mammalian species including rodents, negative feedback regulation of trypsin secretion is mediated by trypsin-sensitive luminal CCK-releasing peptide (LCRF) secreted from the mucosa of the upper intestine into the intestinal lumen in response to dietary components such as amino acids and peptides. LCRF directly stimulates I cells to secrete CCK, with a resulting increase in trypsin secretion from pancreatic acinar cells, and trypsin then degrades LCRF, indicating negative feedback regulation of trypsin-mediated CCK release.

Evidence Supporting this KER

TBD

Biological Plausibility

Regulation of pancreatic secretion

Pancreatic exocrine secretion is controlled mainly by the gastrointestinal hormone cholecystokinin (CCK), which is secreted by CCK-producing I cells located in the mucosa of the small intestine. Multiple mechanisms are involved in the stimulation of CCK release [Wang BJ and Cui ZJ, 2007; Caron J et al, 2017].

Regulation of CCK release mediated by monitor peptide (MP) in rats

In rats, CCK release from I cells in the duodenal mucosa of the small intestine is regulated actively by MP [Miyasaka K et al, 1989a; Fushiki T et al, 1989; Iwai K et al, 1988; Miyasaka K and Funakoshi A, 1998], which consists of 61 amino acids with a molecular weight of approximately 6000. It was first purified from rat pancreatic juice, and its amino acid sequence was subsequently determined [Iwai K et al, 1987].

In the empty intestine, secreted MP is bound to trypsin and thus free MP is maintained at a low level in the intestine; in this situation, CCK release is suppressed. However, after the gastric contents are transported to the small intestine, proteases are postulated to be used for protein hydrolysis, allowing the amount of free MP to increase [Iwai K et al, 1988; Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998; Graf R, 2006]. The increased MP stimulates mucosal I cells to release CCK via their surface MP receptors, stimulating pancreatic exocrine secretion [Liddle RA et al, 1992; Guan D et al, 1990; Cuber JC et al, 1990]. MP binds to the surface of CCK-immunoreactive mucosal cells in the small intestine [Yamanishi R et al, 1993a; Yamanishi R et al, 1993b]. After proteolysis of the intestinal contents, the luminal level of free trypsin is increased, which causes the luminal MP level to return to a low level, followed by a decrease in CCK release [Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998; Graf R, 2006].

Another role of MP as a pancreatic secretory trypsin inhibitor (TI)

Similar to other pancreatic soluble TIs, MP forms complexes with trypsin in the empty intestine to prevent auto-injury by trypsin [Lin YZ et al,

1990; Voet D and Voet JG, 1995]. Once TI is ingested, TI–trypsin complexes are formed, and the intestinal level of free MP is increased to stimulate CCK release [Yamanishi R et al, 1993b], increasing the blood CCK level even on an empty intestine. TIs other than MP show no effect on CCK release [Miyasaka K, 1989a; Tsuzuki S, 1991].

Effects of TIs on MP-mediated CCK release

In contrast, once TIs are ingested, the intestinal concentration of MP is increased due to inhibition of its binding with trypsin and degradation, and the increased MP directly stimulates I cells to release CCK into the blood. In turn, the increased CCK stimulates pancreatic acinar cells to secrete MP as well as pancreatic enzymes, and the secretion of MP further upregulates CCK release via a positive feedback mechanism, especially under trypsin inhibition [Wang BJ and Cui ZJ, 2007; Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998; Liddle RA, 1995].

Some studies have reported that intraduodenal injection of MP stimulates CCK release in rats with external biliary and pancreatic fistulae [Miyasaka K et al, 1989a; Longnecker DS, 1987].

Raw soya flour containing TIs and protease inhibitors such as camostat directly inhibit trypsin activity, and rats treated with these agents showed an increased blood level of CCK [Liddle RA et al, 1984; Goke B et al, 1986; Calam J et al, 1987; Douglas BR et al, 1989; Cuber JC et al, 1990; Playford RJ et al, 1993; Obourn JD et al, 1997; Tashiro M et al, 2004; Komarnytsky S et al, 2011] . The mechanism underlying the increase in CCK release by TIs is thought to involve an increase in the intestinal MP level resulting from trypsin inhibition [Iwai K et al, 1988; Cuber JC et al, 1990; Miyasaka K et al, 1989a].

CCK

CCK is a peptide hormone secreted by I cells located in the mucosa of the small intestine, and it regulates pancreatic exocrine secretion. CCK is secreted as peptide prohormone consisting of 150 amino acids. Several CCK isoforms exist, composed of different numbers of amino acids due to post-transcriptional modifications, although the amino acid sequence of the C-terminal end is common among these isoforms [Rehfeld JF, 2017; Wang BJ and Cui ZJ, 2007].

In addition, MP receptors are thought to be expressed on I cells, based on the findings that MP binds to CCK-positive cells in the mucosa of the small intestine, and this binding is inhibited by TIs [Yamanishi R et al, 1993a; Yamanishi R et al, 1993b].

Empirical Evidence
MP at concentrations ranging from 3 x 10^{-12} to 3 x 10^{-8} M stimulated CCK release from isolated mucosal cells from the rat duodenum in a dose-dependent manner with highest level at 15 minutes after stimulation [Bouras EP et al, 1992].

MP at a concentration range of 2–12 µg/mL induced within a few minutes a dose-dependent transient increase in portal CCK-like immunoreactivity in isolated vascularly perfused rat duodenum/ jejunum [Cuber JC et al, 1990].

In rats with biliary and pancreatic fistula, duodenal infusion of MP at 0.9 µg/rat increased pancreatic secretion and the plasma CCK level [Miyasaka K et al, 1989a].

Sorted CCK-positive rat intestinal mucosal cells stimulated with 30nM MP increased the secretion of CCK in a time-dependent manner starting at 5 min after the start of MP incubation [Liddle RA et al, 1992].

Uncertainties and Inconsistencies
TBD

Quantitative Understanding of the Linkage

TBD

Response-response relationship
MP at concentrations ranging from 3 x 10^{-12} to 3 x 10^{-8} M stimulated mucosal cells isolated from the rat duodenum to release CCK in a dose-dependent manner [Bouras EP et al, 1992].

MP at a concentration range of 2–12 µg/mL induced a dose-dependent transient increase in portal CCK-like immunoreactivity in isolated vascularly perfused rat duodeojejunum MP at 36 µg/mL showed lower CCK release [Cuber JC et al, 1990].

Time-scale
MP stimulated CCK release from isolated mucosal cells from the rat duodenum, sorted CCK-positive rat intestinal mucosal cells, or isolated vascularly perfused rat duodenum/jejunum after or within several minutes from the incubation [Liddle RA et al, 1992; Bouras EP et al, 1992; Cuber JC et al, 1990].

Known modulating factors
In addition to by MP in rats, CCK release from duodenal I cells is stimulated by gastric contents containing fatty acids and amino acids, either directly by specific receptors such as Ca-sensing receptors and the G protein-coupled receptor GPR93 or indirectly by luminal CCK-releasing factors (LCRF) in rats and humans[Caron J et al, 2017]. In humans, LCRF is released from intestinal mucosal cells in response to amino acids and fatty acids, and the LCRF mediate negative feedback regulation of CCK release via LCRF degradation by trypsin [Wang BJ and Cui ZJ, 2007].

Known Feedforward/Feedback loops influencing this KER
In rodents, monitor peptide, a pancreatic secretory trypsin inhibitor, is secreted by pancreatic acinar cells along with trypsin and other digestive enzymes stimulated by CCK [Iwai K et al, 1988; Tsuzuki S et al, 1991]. Because MP binds tightly to trypsin [Voet D and Voet JG, 1995], trypsin inhibition increases the intraluminal concentration of MP in a positive feedback manner [Liddle RA et al, 1984; Wang BJ and Cui ZJ, 2007].

Meanwhile, in mammalian species including rodents, TIs might stimulate CCK release into the bloodstream via an increased luminal concentration of trypsin-sensitive CCK-releasing peptides secreted by duodenal mucosal cells [Miyasaka K et al, 1989c; Lu L et al, 1989; Guan D et al, 1990; Owyang C, 1994; Liddle RA, 1995; Spannagel AW et al, 1996; Herzig KH et al, 1996; Miyasaka K and Funakoshi A, 1998; Marchbank T et al,

1998; Li Y et al, 2000; Owyang C, 1999; Wang Y et al, 2002] . Increased blood level of CCK does not stimulate further secretion of LCRF different from the positive feedback regulation of CCK release by MP.

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Relationship: 2030: Increased blood CCK level leads to Increased acinar cell exocrine secretion (https://aopwiki.org/relationships/2030)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Trypsin inhibition leading to pancreatic acinar cell tumors (https://aopwiki.org/aops/316)	**adjacent**	**High**	**High**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Macaca fascicularis	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Species differences in the mechanism of CCK release

Pancreatic exocrine secretion is controlled mainly by CCK released into the blood steam from intestinal mucosal I cells of the small intestine in response to the gastric contents transported to the intestine [Singer MV and Niebergall-Roth E, 2009; Rehfeld JF, 2017]. Peptides released from gastrointestinal digestion, along with fatty acids, are the main stimuli of CCK release involving several direct and indirect pathways [Caron J et al, 2017].

In humans and canines, amino acids and fatty acids in the gastric contents transported to the small intestine play a major role in stimulating CCK release, which regulates pancreatic exocrine secretion, but MP is not involved in exocrine regulation [Wang BJ and Cui ZJ, 2007]. CCK-stimulated pancreatic exocrine secretion seems to be regulated with negative feedback manner via LCRF.

In rats, however, different from other mammalian species, nutrient protein and protein hydrolysate stimulate CCK release and MP secreted by pancreatic acinar cells plays an active role in protein/protein hydrolysate-stimulated CCK release [Iwai K et al, 1988; Fushiki T et al, 1989]. Ingestion of trypsin inhibitors increases the intestinal level of MP, especially after all nutrient protein is digested in the intestines, causing a subsequent increase in the blood level of CCK. The increased CCK level stimulates pancreatic exocrine secretion of proteins including MP, which in turn further increases the release of CCK. This positive feedback response associated with MP secretion might lead to continuously elevated plasma levels of CCK [Liddle RA, 1995].

Species differences in CCKs

Several isoforms of CCK, including CCK-83, -58, -39, -33, -22, and -8, have been identified, and there are species differences in CCK isoforms (e.g., CCK-33, -22 and -58 are expressed in humans, CCK-58 in dogs, CCK-8, -33 and -58 in cats, CCK-22, -58, -3 and -8 in pigs, CCK-22 and -8 in rabbits, and CCK-58 in rats). All of these CCK isoforms have a highly conserved region of amino acids, and all are ligands of CCK1 receptors [Wang BJ and Cui ZJ, 2007].

Species differences in pancreatic exocrine secretion

In rats, CCK stimulates pancreatic exocrine secretion and acinar cell growth directly via CCK1 receptors expressed on the cell surface, and exocrine secretion is also innervated by vagal afferent nerves expressing CCK1 receptors [Singer MV and Niebergall-Roth E, 2009; Pandiri AR, 2014; Yamamoto M et al, 2003].

On the other hand, human pancreatic acinar cells do not express CCK1 receptors, and CCK-mediated exocrine secretion is regulated exclusively by innervation of vagal nerves expressing CCK1 receptors [Soudah HC et al, 1992; Beglinger C et al, 1992; Singer MV and Niebergall-Roth E, 2009], although there is some evidence of direct stimulation of exocrine secretion of human pancreatic acinar cells [Murphy JA et al, 2008].

Species differences in CCK receptors

CCK1 and CCK2 receptors are expressed in various organs and tissues including digestive and nervous systems, and there are species differences in distribution and expression levels of the receptors.

In rats, pancreatic acinar cells express mainly CCK1 receptors and no CCK2 receptors [Bourassa J et al, 1999]. CCK1 receptors are also expressed in vagal afferent nerve fibers of the gastroduodenal tract. Stimulation of the vagal nerve via CCK1 receptors as well as via physical stimulation by stomach wall distention from ingested food also promotes pancreatic exocrine secretion [Dufresne M et al, 2006].

In humans, on the other hand, CCK2 receptors are dominantly expressed in pancreatic acinar cells, with low expression of CCK1 receptors [Nishimori I et al, 1999]. Ji reported the following: 1) the mRNA level of the CCK2 receptor is higher than that of the CCK1 receptor in the human pancreas; 2) an in situ hybridization experiment showed no expression of either receptor type in the human pancreas, and 3) human pancreatic cells did not show any response to the CCK1 receptor agonist CCK8 or the CCK2 receptor agonist gastrin in vitro [Ji B et al, 2001]. Therefore, human pancreatic acinar cells respond to CCK more weakly compared with the response in rodents.

Although the distribution of CCK receptors is different between humans and rodents, the structures of CCK1 receptors are highly conserved among mammalian species, with 98% homology between rats and mice, 90% between rats and humans, 98% between cynomolgus monkeys and humans, and 89% between dogs and humans [Wang BJ and Cui ZJ, 2007].

Key Event Relationship Description

Pancreatic exocrine secretion is regulated mainly by cholecystokinin (CCK) released by CCK-producing I cells located in the mucosa of the upper small intestine. CCK stimulates exocrine secretion directly via CCK receptors expressed on acinar cell surfaces or indirectly via vagal afferent nerves expressing CCK receptors.

There are two types of CCK receptors: CCK1 (CCK-A) and CCK2 (CCK-B or gastrin) receptors. The former shows high affinity to CCK and the latter to both CCK and gastrin [Wang BJ and Cui ZJ, 2007; Dufresne M et al, 2006].

There are species differences in CCK-mediated pancreatic exocrine secretion. In rats, exocrine secretion from pancreatic acinar cells is regulated directly by CCK1 receptors expressed on the surface of acinar cells and indirectly by vagal afferent nerves expressing CCK1 receptors. Meanwhile, in humans, pancreatic exocrine secretion is regulated mainly by vagal afferent nerves expressing CCK1 receptors [Wang BJ and Cui ZJ, 2007].

The major function of pancreatic exocrine secretion is the production and secretion of digestive enzymes. Zymogen granules located at the apical site of pancreatic acinar cells contain the precursors of multiple digestive enzymes such as trypsinogen, chymotrypsinogen, proesterases, procarboxypeptidase A and B, as well as pancreatic lipase and amylase α. These precursors are secreted by acinar cells into the small intestine, where they are activated by pepsins and peptidases [Berg JM et al, 2002].

Evidence Supporting this KER

TBD

Biological Plausibility

Pancreatic exocrine secretion

The major function of pancreatic exocrine secretion is the release of digestive enzymes, fluid, and bicarbonate in response to food intake. Zymogen granules located at the apical site of pancreatic acinar cells contain the precursors of multiple digestive enzymes, such as trypsinogen, chymotrypsinogen, proesterase, procarboxypeptidase A and B, as well as pancreatic lipase and amylase α. These precursors are secreted into the small intestine, where trypsinogen is converted to trypsin by enteropeptidase, and the newly generated trypsin activates more trypsinogen molecules and other proenzymes [Berg JM et al, 2002].

Regulation of pancreatic exocrine secretion via CCK and CCK receptors

Pancreatic exocrine secretion is regulated mainly by CCK released from CCK-producing I cells located within the mucosa of the small intestine. CCK stimulates exocrine secretion either directly via CCK receptors expressed on acinar cells or indirectly by the vagovagal reflex via CCK receptors. There are species differences in these CCK regulatory mechanisms [Singer MV and Niebergall-Roth E, 2009; Chandra R and Liddle RA, 2009].

CCK receptor subtypes

There are two types of CCK receptors: CCK1 (CCK-A) and CCK2 (CCK-B or gastrin receptor) receptors. The CCK1 receptor exhibits high affinity to all CCK isoforms, whereas the CCK2 receptor exhibits affinity to both CCKs and gastrin [Dufresne M et al, 2006; Rehfeld JF, 2017].

Direct and indirect innervation-mediated regulation of exocrine secretion from acinar cells via CCK receptors

In rats, pancreatic acinar cells express mainly CCK1 receptors, and blood CCK directly stimulates exocrine secretion and acinar cell proliferation [Dufresne M et al, 2006]. Moreover, the vagal afferent nerves also stimulate pancreatic exocrine secretion; CCK stimulates CCK1 receptors expressed on the vagal afferent nerve fibers of the vago–vagal loop, and the acetylcholine generated acts on M3 muscarinic cholinergic receptors to promote pancreatic exocrine secretion [Bourassa J et al, 1999; Adler G, 1997; Ji B et al, 2001; Li Y et al, 1997; Owyang C, 1996].

In humans, the density of CCK receptors expressed on acinar cells is lower than that in rodents, whereas CCK2 receptors are dominantly expressed. Therefore, the responses of acinar cells to CCK seem to be weaker compared with rodents, and pancreatic exocrine secretion in humans is regulated mainly by vagal afferent nerves expressing CCK1 receptors [Wang BJ and Cui ZJ, 2007; Owyang C, 1996; Pandiri AR, 2014].

Empirical Evidence

In rats, diversion of bile pancreatic juice induced more than ten-times increase in plasma concentration of CCK at the end of two hours diversion and caused rapid and sustained increase in pancreatic protein secretion with more than two folds at 60 minutes of diversion compared with the basal levels [Li Y and Owyang C, 1994].

Repeated injections of CCK at 1390 IU s.c. for 3 weeks significantly increased the pancreatic levels and secretion of amylase and trypsin during stimulation with 60 IU/kg-hour of CCK. Peak secretion rates of the enzymes were obtained 45 minutes after the start of the stimulation [Folsch UR et al, 1978].

Trypsin-mediated feedback control of pancreatic enzyme secretion has also been observed in humans.

Intraduodenal perfusion of phenylalanine at 10mM, 5mL/min induced a several times increase in the plasma level of CCK within 15 minutes and a four-times increase in one-hour pancreatic outputs of trypsin and chymotrypsin. Simultaneous intraduodenal perfusion of trypsin with phenylalanine lowered plasma CCK level at 24% and pancreatic output of chymotrypsin at 63% compared with the perfusion of phenylalanine alone. Moreover, intravenous infusion of CCK-8 at 20 and 40 ng/kg/h for 60 minutes showed a dose-dependent increase in pancreatic output of chymotrypsin [Owyang C et al, 1986].

Uncertainties and Inconsistencies

TBD

Quantitative Understanding of the Linkage

TBD

Response-response relationship
CCK action on the stimulation of pancreatic secretion is dose dependent. Doses of CCK that induce physiological concentrations of plasma CCK (up to ~10 pM) stimulate the vagal afferent pathway, whereas doses that produce supraphysiological CCK levels act to stimulate intrapancreatic neurons and pancreatic acini. The brief summaries are as follows:

Intravenous infusion of CCK-8 at 20 and 40 pM/kg/hour or high affinity CCKR agonist CCK-JMV-189 at 22, 44 and 88 µg/kg/hour in rats induced dose-dependent increases in pancreatic protein secretion from 15 minutes of infusion, which was blocked by the CCK1 receptor antagonist L-364,718 [Li Y et al, 1997].

Physiological level of plasma CCK (up to ~10 pM) result in stimulation of the vagal afferent pathway originating from the gastroduodenal mucosa, whereas doses that induce supraphysiological CCK levels result in stimulation of intrapancreatic neurons and pancreatic acini [Owyang C, 1996].

Time-scale
In rats in which bile and pancreatic juice had been returned to the duodenum, intraduodenal administration of 30 mg RSF stimulated a 1-h integrated increase in pancreatic protein output of 2.2 ± 1.1 mg/h (mean ± SE) [Jordinson M et al, 1996].

Bile-pancreatic juice diversion in rats increases pancreatic protein secretion with more than two fold 60 minutes after the start of diversion with elevated blood level of CCK [Li Y and Owyang C, 1994].

Intravenous infusion of CCK at 60 IU/kg/hour induces the pancreatic secretion of amylase and trypsin with peak level at 45 minutes after the start of the stimulation [Folsch UR et al, 1978].

In human intraduodenal perfusion of phenylalanine at 10mM, 5mL/min induced a several times increase in the plasma level of CCK within 15 minutes and a four-times increase in one-hour pancreatic outputs of trypsin and chymotrypsin. Intravenous infusion of CCK-8 at 20 and 40 ng/kg/h for 60 minutes showed a dose-dependent increase in pancreatic output of chymotrypsin [Owyang C et al, 1986].

These results suggest that CCK-induced pancreatic exocrine secretion occur within a short time after CCK infusion or stimulation of CCK release.

Known modulating factors
Disruption of the CCK1 receptor in rats also affects pancreatic exocrine secretion [Miyasaka K et al, 1998].

Capsaicin and atropine inhibit cholinergic vagus nerve reflexes to reduce CCK-mediated pancreatic enzyme secretion [Li Y et al, 1997; Yamamoto M et al, 2003; Li Y and Owyang C, 1994; Soudah HC et al, 1992; Owyang C et al, 1986].

Known Feedforward/Feedback loops influencing this KER
TBD

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Relationship: 2031: Increased acinar cell exocrine secretion leads to Acinar cell proliferation (https://aopwiki.org/relationships/2031)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Trypsin inhibition leading to pancreatic acinar cell tumors (https://aopwiki.org/aops/316)	**adjacent**	**High**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Macaca fascicularis	Macaca fascicularis	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

The effect of CCK on acinar cell proliferation differs between rodents and humans.

In rats, CCK stimulates pancreatic exocrine secretion directly via CCK1 receptors expressed on the cell surface and also via innervation of afferent vagal nerves expressing CCK1 receptors [Singer MV and Niebergall-Roth E, 2009; Pandiri AR, 2014]. Higher plasma levels of CCK might also directly stimulate acinar cell proliferation via surface CCK receptors [Yamamoto M et al, 2003].

In contrast to rats, monkeys receiving repeated doses of the CCK1 receptor agonist GI181771X for up to 52 weeks showed no hypertrophy or histopathological changes in the pancreas [Myer JR et al, 2014]. Regarding humans, obese patients treated with GI181771X for 24 weeks showed no abnormal changes in the pancreas by ultrasonography or MRI [Jordan J et al, 2008]. Moreover, some epidemiological surveys suggested that long-term ingestion of TI-containing foods does not increase the risk of pancreatic cancer [Miller RV, 1978], although oral ingestion of raw soya flour containing TIs was reported to stimulate CCK release in humans [Calam J et al, 1987].

These findings suggest that exocrine secretion in humans and primates is regulated exclusively by innervation of vagal afferent nerves expressing CCK1 receptors [Soudah HC et al, 1992; Beglinger C et al, 1992; Singer MV and Niebergall-Roth E, 2009], with little effect on acinar cell proliferation, although the possibility of direct stimulation of exocrine secretion from human pancreatic acinar cells has been suggested [Murphy JA et al, 2008].

Meanwhile, a strong relationship between pancreatic cancers and a history of subtotal gastrectomy [Mack TM et al, 1986], which induced a higher plasma CCK level in response to fat [Hopman WP et al, 1984], was reported. Therefore, the effect of CCK on acinar cell proliferation in humans is controversial.

Key Event Relationship Description

In rats, an increased blood level of CCK stimulates pancreatic acinar cells to secrete digestive enzymes directly via surface CCK1 receptors and indirectly via innervation of vagal afferent nerves expressing CCK1 receptors. A persistent increase in the blood CCK level stimulates pancreatic acinar cell proliferation directly via surface CCK1 receptors. On the other hand, human pancreatic acinar cells express CCK2 receptors, which do not respond to CCK in terms of secretion and proliferation. Pancreatic enzyme secretion in humans is innervated by afferent vagal nerves expressing CCK1 receptors; however, its involvement in acinar cell proliferation is unclear.

Evidence Supporting this KER

TBD

Biological Plausibility

CCK-induced pancreatic acinar cell proliferation

An increased plasma level of CCK directly induces proliferation of pancreatic acinar cells via surface CCK1 receptors as well as exocrine secretion in rodents. Consuming raw soya flour for 30 days, administration of trypsin inhibitor in drinking water for 7 days, or repeated injection of cholecystokinin for 7 days induced pancreatic hypertrophy and hyperplasia [Yanatori Y and Fujita T, 1976]. Repeated administration of CCK for 21 days [Folsch UR et al, 1978] and treatment with the CCK8 and CCK1 receptor agonist A-71623 for 3 weeks [Povoski SP et al, 1994] also induced pancreatic hyperplastic changes in mice [Tashiro M et al, 2004]. Addition of 0.1% camostat in the diet for 10 days increased pancreatic weight and protein and DNA levels in a time-dependent manner in mice [Tashiro M et al, 2004].

The CCK1 receptor agonist GI181771X induced pancreatitis due to abnormal basolateral secretion of Zymogen granules at the high dose and acinar cell hypertrophy at the middle and low doses in ratsan. The author mentioned JAK1/2–STAT1/3 activation leading to p38MAPK activation as a mechanism underlying acinar cell proliferation.

Direct effect of CCK on acinar cell proliferation via CCK receptors

In rats, the trypsin inhibitor FOY-305 increased pancreatic weight and induced acinar cell hypertrophy, and denervation of vagal nerves had little effect on these hypertrophic changes [Aki T et al, 1989]. Administration of CCK-8 at physiological doses induced exocrine secretion, and atropine and vagal nerve denervation suppressed this exocrine secretion but not that induced by non-physiological doses of CCK-8 [Li Y and Owyang C, 1993]. These results suggest that the involvement of vagal nerve innervation in acinar cell proliferation under an increased blood CCK level might be low, and this may also be the case in humans, but the evidence is unclear [Chandra R and Liddle RA, 2009].

Empirical Evidence

KE3/KE4

In rats fed 20, 40, or 100% RSF-containing diet for up to 36 weeks, pancreatic hypertrophy was found in all RSF-fed groups, whereas hyperplasia was found only in the 40 and 100% RSF-fed groups [Crass RA and Morgan RG, 1982].

KE3

In rats in which bile/pancreatic juice had been returned to the duodenum, intraduodenal administration of 30 mg RSF increased the total amount of 1-h pancreatic protein output at 2.2 ± 1.1 mg/h (mean ± SE) with highest CCK levels at 30 or 40 minutes after RSF administration [Jordinson M et al, 1996].

KE4

In rats, administration of TIs in drinking water (“Trypsin soybean inhibitor” (Miles), 400mg/100mL) or injection of CCK (CCK-PZ or CCK-33,400 Ivy Dog unit) for 7 days increased acinar cell proliferation as well as acinar cell hypertrophy [Yanatori Y and Fujita T, 1976], and RSF feeding at libitum increased acinar cell proliferation from 7 to 28 days of treatment leading to hypertrophy and hyperplasia [Oates PS and Morgan RG, 1984].

These results showed that trypsin inhibition-induced acinar cell proliferation (hyperplasia) developed at higher TI doses compared with the development of pancreatic hypertrophy caused by increased secretion, and that pancreatic exocrine secretion and increased acinar cell proliferation were found at 1 h and 7 days, respectively, after the start of TI or CCK treatment.

Uncertainties and Inconsistencies

A8947, a broadleaf herbicide with trypsin inhibitory action, was fed to male rats for up to 28 days, at doses of 0, 300, 10,000, and 30,000 ppm. A8947 at 10,000 and 30,000 ppm induced significant increases in acinar cell proliferation after 7 days, followed by a decrease to control levels by 28 days [Obourn JD et al, 1997]. The reason why the TI-induced increase in acinar cell proliferation is transient is unclear.

In humans, the involvement of innervation of vagal nerves in acinar cell proliferation under an increased blood level of CCK might be low, but this is unclear [Chandra R and Liddle RA, 2009].

Quantitative Understanding of the Linkage

TBD

Response-response relationship

KE3 and KE4 in rats injected with CCK

In rats repeatedly injected subcutaneously with CCK at 7.5 or 30 Ivy dog units (IU) twice daily for 20 days, pancreatic wet weight and DNA content / 100g BW increased with a same manner compared with saline-treated rats, however, pancreatic output of amylase and trypsin in response to submaximal intravenous stimulation with CCK at 15 IU/kg/hour increased with dose-dependent manner. [Folsch UR et al, 1978].

KE3 and KE4 in rats treated with TIs

A8947, a broadleaf herbicide with trypsin inhibitory action, was fed to male rats for up to 28 days, at doses of 0, 300, 10,000, and 30,000 ppm, or 56 days, at 0 and 30,000 ppm. A8947 at 10,000 and 30,000 ppm induced significant increases in pancreatic weight, acinar cell proliferation, diffuse acinar cell hypertrophy, and the plasma CCK level after 7 days. The increases in pancreatic weight and the CCK level were maximum at day 14 and then maintained throughout the study, whereas acinar cell proliferation peaked at day 7 but then decreased to control levels by day 28 [Obourn JD et al, 1997]. MK-329, a specific CCKA receptor antagonist, completely abolished the increase in pancreatic weight induced by 30,000 ppm A8947 after 7 days [Obourn JD et al, 1997].

Weanling male Wistar rats were fed 15 diets consisting of four concentrations of purified soybean TIs (93, 215, 337, and 577 mg/100 g diet) and three protein concentrations (10%, 20%, and 30%), as well as raw and heat-treated soy flour containing 10% protein. Rats were sacrificed at 3-month intervals, starting at 6 months, over a period of 22 months [Rackis JJ et al, 1985]. Trypsin and chymotrypsin activities per 100g BW, RNA and DNA contents of pancreas indicative of pancreatic hypertrophy and hyperplasia, respectively, were already increased in all of the TI and protein-fed animals after 6-month dosing, although pancreatic nodules were increased in number at 15 months of dosing or later at 215 mg TI/100 g diet or higher [Liener IE et al, 1985].

Time-scale

In rats in which bile and pancreatic juice had been returned to the duodenum, intraduodenal administration of 30 mg RSF stimulated a 1-h integrated increase in pancreatic protein output of 2.2 ± 1.1 mg/h (mean ± SE) [Jordinson M et al, 1996].

Pancreatic hypertrophy was observed in rats fed an RSF-containing diet within 9 days [Rackis JJ, 1965; Watanapa P and Williamson RC, 1993].

Rats fed RSF showed a biphasic increase in acinar and duct cell proliferation, as determined by [3H]-thymidine incorporation into pancreatic DNA, on days 2–4 and again on days 7–28 after the start of RSF feeding. The first peak in DNA synthesis may represent a regenerative response to tissue damage. The second more delayed peak appears to represent the development of hyperplasia in response to a trophic stimulus [Oates PS and Morgan RG, 1984].

Rats administered TIs in drinking water for 7 days or repeatedly injected with CCK for 7 days [Yanatori Y and Fujita T, 1976] exhibited mitotic figures in the acinar, centroacinar, and intercalated portions of the pancreas and in excretory duct cells, as well as marked pancreatic hypertrophy [Oates PS and Morgan RG, 1984].

A8947, a broadleaf herbicide with trypsin inhibitory action, was fed to male rats for up to 28 days, at doses of 0, 300, 10,000, and 30,000 ppm. A8947 at 10,000 and 30,000 ppm induced significant increases in acinar cell proliferation after 7 days, followed by a decrease to control levels by 28 days [Obourn JD et al, 1997].

In the abovementioned studies [Rackis JJ et al, 1985; Liener IE et al, 1985], the increases in exocrine activity and acinar cell hyperplasia and hypertrophy were found at the earliest sacrifice (6 months). The exocrine activities and hypertrophic changes remained unchanged thereafter, whereas the hyperplastic changes became more pronounced until the final sacrifice (22 months).

These findings show that pancreatic exocrine secretion and increased acinar cell proliferation were found at 1 h and 7 days, respectively, after the start of TI or CCK treatment.

CCK was released within 1 h after intraduodenal administration of RSF, and acinar cell proliferation was elevated approximately 7 days after the start of RSF feeding, although some TIs induced transient acinar cell proliferation within 7 days as a regenerative change to acute pancreatic injury.

Known modulating factors

TIs including RSFs are reported to induce pancreatic acinar cell proliferation as well as acinar cell hypertrophy due to increased pancreatic protein secretion in rats. Administration of CCK receptor agonist and CCK also induce acinar cell hyperplasia and hypertrophy as follows.

Acinar cell changes induced by a CCK receptor agonist

A novel CCK1 receptor agonist, GI181771X, was administered to mice and/or rats at doses of 0.25–250 mg/kg/day from 7 days to 26 weeks, and pancreatic acinar cell responses were examined. The treated animals showed a wide range of morphological changes in the pancreas that were dose and time dependent, including necrotizing pancreatitis, acinar cell hypertrophy/atrophy, zymogen degranulation, focal acinar cell hyperplasia, and interstitial inflammation [Myer JR et al, 2014].

Acinar cell proliferation in rats injected with CCK

Rats 1) fed raw soybeans for 30 days, 2) administered TIs in drinking water for 7 days, or 3) repeatedly injected with CCK for 7 days exhibited increased mitotic figures in the acinar, centroacinar, and intercalated portions of the pancreas and in excretory duct cells, as well as marked pancreatic hypertrophy [Myer JR et al, 2014].

Known Feedforward/Feedback loops influencing this KER
TBD

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Relationship: 2032: Acinar cell proliferation leads to Acinar cell tumors (https://aopwiki.org/relationships/2032)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Trypsin inhibition leading to pancreatic acinar cell tumors (https://aopwiki.org/aops/316)	**adjacent**	**High**	**High**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Macaca fascicularis	Macaca fascicularis	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Mus musculus	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Rats fed a diet supplemented with soy and potato TI concentrates for 28 days developed pancreatic hypertrophy, and after long-term feeding (95 weeks), the rats developed nodular hyperplasia and acinar adenoma in a dose-dependent manner. Although mice responded similarly to rats to soy TIs in short-term (28 days) feeding experiments, they did not form these pathologies (hyperplasia or acinar adenoma) following long-term feeding. This considerable species difference suggests that the propensity to develop preneoplastic and neoplastic lesions in the pancreas is not predicted by short-term pancreatic hypertrophic and hyperplastic responses to TIs [Gumbmann MR et al, 1989].

The effects of TI-containing diets were evaluated in rats, mice, and hamsters for 30 weeks. In rats and mice, pancreatic weight and DNA, RNA, and protein levels increased in response to a diet consisting of RSF (which contains TIs). Only rats fed RSF developed reversible micro- and macro-nodules after 6 months of treatment, and longer treatment with RSF resulted in further growth in the pancreas and, ultimately, development of adenomas and carcinomas from pancreatic acinar cells [McGuinness EE et al, 1985].

The reasons for the abovementioned species differences in tumor outcome based on hyperplastic changes in acinar cells are unclear, even in rodents.

Meanwhile, a strong relationship between pancreatic cancer and a history of subtotal gastrectomy [Mack TM et al, 1986], which induced a higher plasma CCK level in response to fat [Hopman WP et al, 1984], was reported. On the other hand, some epidemiological surveys suggested that long-term ingestion of TI-containing foods does not increase the risk of pancreatic cancer [Miller RV, 1978], although oral ingestion of raw soya flour containing TIs was reported to stimulate CCK release in humans [Calam J et al, 1987]. Therefore, the effect of CCK on acinar cell proliferation in humans is controversial.

In cases where acinar cell proliferation is enhanced due to a certain treatment, the risk of acinar cell tumor formation may be high in humans as well as rodents.

Key Event Relationship Description

An increased blood level of CCK is the main factor responsible for a sustained increase in acinar cell proliferation and subsequent tumor formation.

Evidence Supporting this KER

TBD

Biological Plausibility

Trypsin inhibitor-induced pancreatic tumor formation

Ingestion of raw soya flour, which contains trypsin inhibitory activity, by rats for 2 years induced pancreatic hypertrophy due to acinar cell hyperplasia and acinar cell tumors [Rackis JJ et al, 1985; Woutersen RA et al, 1991]. Rats given raw soya flour or the trypsin inhibitor camostat exhibited pancreatic hypertrophy and acinar cell hyperplasia, and rats administered the pancreatic carcinogen azaserine followed by camostat exhibited acinar cell tumor formation [Gumbmann MR et al, 1986; Lhoste EF et al, 1988; Bell RH Jr et al, 1992].

Promotion of pancreatic acinar cell tumors via CCK

In addition, the suggestion that trypsin inhibition-induced pancreatic acinar cell tumor formation is promoted by increased acinar cell proliferation via CCK receptors is supported by the following study.
After initiating treatment with 30 mg/kg azaserine at 19 days of age, rats were treated with camostat, CCK8, or gelatin control, in combination with or without the CCK receptor antagonist CR-1409 (once daily, 3 days/week for 16 weeks). After 16 weeks, both camostat and CCK8 stimulated pancreatic growth and the development of azaserine-induced acidophilic putative preneoplastic foci. CR-1409 almost completely abolished the effect of CCK8 and significant attenuated the effect of camostat [Douglas BR et al, 1

Soybean trypsin inhibitor

KE4/AO:

Soy and potato trypsin inhibitor (TI) concentrates were prepared from defatted raw soy flour and potato juice. Rats and mice were fed a diet supplemented with each concentrate to provide 100 and 200 mg of trypsin inhibitor activity per 100 g of diet. In short-term (28 d) experiments in rats, both sources of TI induced pancreatic hypertrophy (KE4). After long-term feeding (95 weeks) in rats, soy and potato TI induced dose-related increases in pancreatic nodular hyperplasia and acinar adenoma (AO) [Gumbmann MR et al, 1989].

Rats were continuously fed diets containing lower amounts of raw soya flour (RSF, 5%, 25% and 50%) with weekly intraperitoneal injection of either azaserine at 5mg/kg BW or saline for up to 85 weeks or were fed RSF intermittently (2 days per week).After a maximum of 2 years of study, continuous feeding of as little as 5% RSF developed pancreatic micro/macroscopic nodules and stimulated the development of azaserine-initiated nodular hyperplasia and tumorigenesis. Intermittent feeding of 25 , 50 and 100% RSF also induced nodular hyperplasia. In addition, consuming a 100% RSF diet for 2 days per week resulted in the development of pancreatic cancer in some of the rats [McGuinness EE and Wormsley KG, 1986].

Protease inhibitor camostat:

KE4:

Adult Fischer 344 (F344) and Lewis rats fed camostat mixed in the diet to define a level that induced pancreatic hypertrophy and hyperplasia. As little as 0.02% fed 3 days per week was effective [Lhoste EF et al, 1988].

AO:

F344 rats were injected s.c. twice with azaserine at 30 mg/kg BW and thereafter were given camostat at 200 mg/kg BW by gavage 5 days a week until autopsy 18 weeks later. In addition, azaserine-treated Lewis rats were fed camostat in the diet at 0.5 g/kg diet for 4 weeks and then 0.2 g/kg diet 3 consecutive days a week for 8 or 16 weeks until autopsy. In these experiments the number and size of atypical acinar cell foci and nodules (AACN) were increased in comparison with the control groups. The data suggest a promoting effect of dietary camostat on the growth of azaserine-induced preneoplastic lesions in the pancreas of both rat strains [Lhoste EF et al, 1988].

989].

Sustained pancreatic growth (acinar cell proliferation) leading to acinar cell tumor formation

Rats fed a diet containing raw soya flour developed micro- and macro-nodules. Longer treatment with raw soya flour resulted in further growths in the pancreas and, ultimately, development of adenomas and carcinomas in the acinar pancreas. The pancreatic changes were reversible up to 6 months of consuming the raw soya flour diet but became irreversible thereafter [McGuinness EE et al, 1985].

Empirical Evidence

Soybean trypsin inhibitor

KE4/AO:

Soy and potato trypsin inhibitor (TI) concentrates were prepared from defatted raw soy flour and potato juice. Rats and mice were fed a diet supplemented with each concentrate to provide 100 and 200 mg of trypsin inhibitor activity per 100 g of diet. In short-term (28 d) experiments in rats, both sources of TI induced pancreatic hypertrophy (KE4). After long-term feeding (95 weeks) in rats, soy and potato TI induced dose-related increases in pancreatic nodular hyperplasia and acinar adenoma (AO) [Gumbmann MR et al, 1989].

Rats were continuously fed diets containing lower amounts of raw soya flour (RSF, 5%, 25% and 50%) with weekly intraperitoneal injection of either azaserine at 5mg/kg BW or saline for up to 85 weeks or were fed RSF intermittently (2 days per week).After a maximum of 2 years of study, continuous feeding of as little as 5% RSF developed pancreatic micro/macroscopic nodules and stimulated the development of azaserine-

initiated nodular hyperplasia and tumorigenesis. Intermittent feeding of 25 , 50 and 100% RSF also induced nodular hyperplasia. In addition, consuming a 100% RSF diet for 2 days per week resulted in the development of pancreatic cancer in some of the rats [McGuinness EE and Wormsley KG, 1986].

Protease inhibitor camostat:

KE4:

Adult Fischer 344 (F344) and Lewis rats fed camostat mixed in the diet to define a level that induced pancreatic hypertrophy and hyperplasia. As little as 0.02% fed 3 days per week was effective [Lhoste EF et al, 1988].

AO:

F344 rats were injected s.c. twice with azaserine at 30 mg/kg BW and thereafter were given camostat at 200 mg/kg BW by gavage 5 days a week until autopsy 18 weeks later. In addition, azaserine-treated Lewis rats were fed camostat in the diet at 0.5 g/kg diet for 4 weeks and then 0.2 g/kg diet 3 consecutive days a week for 8 or 16 weeks until autopsy. In these experiments the number and size of atypical acinar cell foci and nodules (AACN) were increased in comparison with the control groups. The data suggest a promoting effect of dietary camostat on the growth of azaserine-induced preneoplastic lesions in the pancreas of both rat strains [Lhoste EF et al, 1988].

Uncertainties and Inconsistencies
TBD

Quantitative Understanding of the Linkage

TBD

Response-response relationship
Hypertrophy/hyperplasia of acinar cells and tumor development in rats fed TI-containing diet were examined in the same rat study reported as follows:

Weanling male Wistar rats were fed 15 diets consisting of four concentrations of purified soybean TIs (93, 215, 337, and 577 mg/100 g diet) and three protein concentrations (10%, 20%, and 30%), as well as raw and heat-treated soy flour containing 10% protein. Rats were first sacrificed at 6 months and at 3-month intervals thereafter over a period of 22 months [Rackis JJ et al, 1985]. In this study, the following dose responses for KE4 and AO were obtained.

KE4:
Hypertrophy and hyperplasia of the pancreas determined by pancreas weight and RNA and DNA content developed at 6 months and were likewise positively correlated with the levels of TI and protein. Although the hypertrophic response remained unchanged, hyperplasia became more pronounced as the period of exposure to TI was prolonged [Liener IE et al, 1985].

AO:
Nodular hyperplasia of acinar cells was observed in the first sacrifice group at 6 months. Incidence of the lesion was positively related to both time of exposure and level of dietary TI. Acinar cell adenoma was first observed at 18 months and was most prevalent in rats fed the highest concentration of TI [Spangler WL et al, 1985].

Time-scale
KE4:

Several studies have suggested that acinar cell proliferation is induced approximately 7 days after treatment with TIs or CCK. Rats fed RSF showed a biphasic increase in the proliferation of acinar and duct cells on days 2–4 and again on days 7–28 after the start of RSF feeding. The first peak may represent a regenerative response to tissue damage. The second more delayed peak appears to represent the development of hyperplasia in response to a trophic stimulus [Oates PS and Morgan RG, 1984]. Rats administered TIs in drinking water for 7 days or repeatedly injected with CCK for 7 days exhibited increased mitotic figures in the acinar, centroacinar, and intercalated portions of the pancreas and in excretory duct cells, as well as marked pancreatic hypertrophy [Yanatori Y and Fujita T, 1976].

AO:

Increased CCK-mediated acinar cell proliferation might lead to acinar cell tumor formation, as shown by the following findings:
In rats fed soybean TIs, acinar cell hyperplasia was observed at the first sacrifice time point (6 months) and became more pronounced with prolonged TI exposure. Nodular hyperplasia of acinar cells was also found at 6 months and increased at later dosing periods. Acinar cell adenomas were first observed at 18 months of TI exposure [Liener IE et al, 1985; Spangler WL et al, 1985].

Morgan et al. reported that rats fed an RSF diet for 24 weeks developed pancreatic hypertrophy and hyperplasia, as determined by DNA, RNA, and protein contents in the pancreas, and developed more pronounced azaserine (30 mg/kg once a week for 5 weeks)-induced nodular hyperplasia compared with rats fed a heat-treated soy flour diet [Morgan RG et al, 1990].

Known modulating factors
Trypsin inhibition promotes acinar cell tumor formation.

TI-enhanced growth of azaserine-induced pancreatic preneoplastic lesions were reduced especially in size by the CCK receptor antagonist lorglumide (CR-1409) [Douglas BR et al, 1989].

Pancreatic growth was induced by cholestyramine, similar to that by TIs, presumably because of the bile salt-binding properties of cholestyramine. This finding suggests that removal of proteases and bile salts from the upper small intestine results in pancreatic growths, which may become neoplastic [McGuinness EE et al, 1985].

The thrombin inhibitor ximelagatran induced focal/multifocal acinar cell hyperplasia and adenomas in the pancreas of rats after 24 months of oral administration at 240 µmol/kg/day. However, in mice, no tumors formed after 18 months of treatment with ximelagatran. Treatment with dabigatran, which is in the same class as ximelagatran, showed no carcinogenicity in mice or rats [Stong DB et al, 2012].

Unsaturated fat (corn oil) was reported to promote the growth of azaserine-induced preneoplastic lesions and acinar cell tumors, without inducing pancreatic hypertrophy, in the rat pancreas [Woutersen RA et al, 1991].

Known Feedforward/Feedback loops influencing this KER
TBD

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OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

令和元年度 分担研究報告書

非遺伝毒性発がんの免疫組織化学染色による評価法確立に関する研究

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研究要旨

非遺伝毒性の発がん機序には、prolactin（PRL）や甲状腺刺激ホルモン（Thyroid stimulating hormone：TSH）、thyroxine（T4）を含む各種ホルモンレベルの変動と関連しているものも多くみられる。近年、経済協力開発機構（Organisation for Economic Co-operation and Development：OECD）では 試験法ガイドライン（TG：Test Guideline）408 のげっ歯類を用いた 90 日間反復投与毒性試験等において、内分泌攪乱作用の有無を検討するため血清中の triiodothyronine（T3）、T4 や TSH などの測定が追加された。変動の意義やヒトへの外挿性を考慮する上でも、これらの指標の重要性が認識されているが、汎用性が高く、より高感度の測定方法、あるいは血清以外を用いたホルモン変動の解析方法が必要と考えられる。平成 31 年度（令和元年度）においては、前年度に作製した血清ホルモン濃度が変動したラットモデルのサンプルにおいて、十分な感度が得られていなかった血清 TSH 値について測定方法を改良した。また、血清 T4 値と免疫組織化学染色による甲状腺の T4 発現動態について比較検討した。その結果、抗体とビーズの複合体の蛍光強度についてフローサイトメーターを用いた TSH の測定により、雌雄の AMT 群及び雌の PTU 群で基礎食群に比べて有意な高値が確認された。また、有意差は認めなかったものの雄の PTU 群及び雌雄の AGT 群についても高値傾向が見られ、下垂体における抗 TSH 抗体を用いた免疫組織化学染色による評価に応用可能な検体が得られた。また、甲状腺における抗 T4 抗体を用いた免疫組織化学染色による検討では、血清での T4 低値を反映する結果が得られるとともに、変動の機序に関する情報も得られる可能性が示唆された。以上の検討法は、今後、他のホルモンの評価についても応用可能と考えられた。

A. 研究目的

非遺伝毒性の発がん機序には、prolactin（PRL）や甲状腺刺激ホルモン（Thyroid stimulating hormone : TSH）、thyroxine（T4）を含む各種ホルモンレベルの変動と関連しているものも多くみられる。経済協力開発機構（Organisation for Economic Co-operation and Development : OECD）において開発が推進されている有害性発現経路（AOP：Adverse Outcome Pathway）では、起始となる標的分子の事象（Molecular Initiating Event, MIE）及びそれに引き続く重要な事象（Key Event、KE）は測定可能であることが必須とされている。よって、毒性試験における血中 PRL、TSH 及び T4 等の発現変動の評価は、非遺伝毒性発がんにおける MIE 又は KE と

しても重要な意味を持っている。更に近年、OECD では 試験法ガイドライン（TG : Test Guideline）408 のげっ歯類を用いた 90 日間反復投与毒性試験等において、内分泌攪乱作用の有無を検討するため血清中の triiodothyronine（T3）、T4 や TSH などの測定が追加された。変動の意義やヒトへの外挿性を考慮する上でも、これらの指標の重要性が認識されている。しかし、通常の方法（ECLIA 等）によるホルモン濃度の測定には 1 項目について 0.4〜0.5 ml の血清が必要であり、毒性評価の為必須である複数項目の血液生化学検査に追加することは、ラットでは可能性があるものの、マウスでは困難である。以上の背景から、汎用性が高く、より高感度の測定方法、あるいは血清以外を用いたホルモン変動の解析方法が必要と考えられる。

本分担研究では、非遺伝毒性の発がん機序に関連する、PRL、TSH、T4 を含む各種ホルモンレベルの簡便な半定量的検出法の確立を目的として、通常の毒性試験で得られる病理標本を用いた免疫組織化学染色による評価法の開発を目指す。平成 31 年度（令和元年度）においては、前年度に作製した血清ホルモン濃度が変動したラットモデルのサンプルにおいて、十分な感度が得られていなかった血清 TSH 値について測定方法を改良した。また、血清 T4 値と免疫組織化学染色による甲状腺の T4 発現動態について比較検討した。

B. 研究方法

平成 30 年度に 6 週齢 SD ラット（雌雄各群 5 匹）をもちいて、基礎食（Basal diet）群、1000 ppm aminotriazole（AMT）飲水投与群、20 ppm vitamin D_3（VD3）混餌投与群、50 ppm propylthiouracil（PTU）飲水投与群、500 ppm phenobarbital（PB）混餌投与群、6000/1500 ppm aminoglutethimide（AGT）混餌投与群、10 ppm estradiol（E2）混餌投与群の 7 群を設定し、29-30 日目にイソフルラン吸入麻酔下にて後大動脈からの採血後、放血し、解剖した。肝臓、腎臓、甲状腺、下垂体、卵巣、精巣及び副腎は重量測定を行い、それらに加え脾臓及び子宮についてホルマリン固定後、パラフィン包埋切片、病理組織学的検討用の HE 標本を作製した。

血清生化学的検査として、平成 30 年度に、主要項目と共に T3、T4、TSH、ACTH、卵胞刺激ホルモン（Follicle stimulating hormone、FSH）、黄体ホルモン（Luteinizing hormone, LH）、PRL、estradiol、progesterone、testosterone などの血清中濃度測定を実施したが、TSH はいずれの検体も検出限界以下とされた。本年度は TSH について、一般財団法人残留農薬研究所の協力を得て Milliplex Map Rat Thyroid Hormone TSH Panel (EMD Millipore, USA) を使用し、蛍光ビーズ法にて測定した。すなわち、それぞれの血清サンプルをビーズおよび検出抗体と混合し、サンプルの蛍光強度をフローサイトメーター (FACSVerse, BD, Tokyo) を用いて測定した。スタンダードにおける蛍光強度の検量線から血清サンプルの TSH 濃度 (ng/mL) を算出した。検体は、1 匹の血清サンプルに対して 2 本の測定用サンプルを用意し、2 本の測定値の平均値を個体ごとの TSH 濃度とした。

また、平成 30 年度の結果から、血清 T4 値の有意な変動が確認されたため、全動物の甲状腺サンプルを用いて T4 について免疫組織化学染色を実施した。標本は脱パラフィン後、抗原の賦活化のためクエン酸緩衝液 pH6.0（関東化学）に浸漬してオートク

レーブで 121℃ 15 分間熱処理を行い、3% H_2O_2 メタノール溶液にて内因性ペルオキシダーゼ阻害処理、10%正常ヤギ血清（ニチレイ）にてブロッキングを行った。1 次抗体には、抗 Thyroxin(T4)マウスモノクローナル抗体（Clone XM212, Dako ; 希釈倍率 : x 2,000）を用いて 4℃ 下で 1 晩反応させ、2 次抗体にはヒストファインシンプルステインラット MAX-PO(M)を 30 分反応させたのち、DAB を用いて発色させた。

統計解析

データは Prism により集計し Basal diet 群を基準とした Dunn の多群検定を行い、$p<0.05$ を有意とした。図標中には、*$p<0.05$、**$p<0.01$ で有意差の程度を示した。

（倫理面への配慮）

投与実験は混餌及び飲水投与が主体であり，動物の苦痛を最小限に留めた。また，動物はすべてイソフルランの吸入麻酔下で大動脈からの脱血により屠殺し，動物に与える苦痛は最小限に留めた。また，動物実験、飼育および管理に当たっては、「国立医薬品食品衛生研究所動物実験に関する指針」に従い、動物の愛護に十分配慮して行った。

C. 研究結果

C.1. 血清 TSH の測定 (Table 1, Figure 1a,1b)

今回の測定においては、前年度検出限界以下とされた検体を凍結保存したサンプルを用いた。3 検体については、保存状態から計測不可となったが、その他の検体は測定値を得ることができた。2 回に分けて測定された測定日ごとの検量線は、相関係数 R^2 が 0.9987 及び 0.9912 であり、直線性を担保していることから、測定の適切性が示された。雌雄の AMT 群及び雌の PTU 群で基礎食群に比べて有意な高値を認めた。また、有意差は認めなかったものの雄の PTU 群及び雌雄の AGT 群についても高値傾向が見られた。

C.2. T4 の免疫組織化学染色による検討 (Figure 2)

前年度に得られた各群の甲状腺組織について抗 T4 抗体を用いた免疫組織化学染色を実施したところ、雌雄の AMT 群および PTU 群では甲状腺の内腔での陽性所見はほとんど認めず、明らかな T4 の染色性低下が見られた。

D. 考察

本分担研究では、非遺伝毒性の発がん機序に関連する、PRL、TSH、T4 を含む各種ホルモンレベルの簡便な検出法の確立を試みている。本年度においては、血清 TSH の測定を抗体とビーズの複合体の蛍光強度についてフローサイトメーターを用いた測定で再検討したところ、十分な結果が得られた。すなわち、スタンダードサンプルでは適切な検量線が得られ、雌雄の AMT 投与群及び雌の PTU 群のサンプルでは有意な高値を認めた。これらの群では前年の検討において血清 T3 及び T4 の有意な低値が確認されており、そのネガティブフィードバックによって下垂体からの TSH 産生が高まるとされるこれらの化合物の既知の毒性所見が反映されていた。1 群あたりの検体数が 5 と限られていることから有意差はないものの、雄の PTU 群においても TSH 値の高値傾向が見られた。一方、雌のみが有意な T3 の低値を示していた AGT 群においては、雌雄で高値傾向がみられた。TSH については、現在の所、免疫組織化学染色による検出方法が

確立できておらず、引き続き検討を継続する必要がある。

また、前年の検討において、血清 T4 の低値が明らかであった個体を含む全個体の甲状腺について T4 の免疫組織化学染色を実施したところ、雌雄の AMT 群および PTU 群では明らかな T4 の染色性低下が見られ、ホルモンレベルの変動を毒性試験において恒常的に採取検討される甲状腺の病理標本を用いてスクリーニングできる可能性が示唆された。一方で、T4 の有意な高値を示した雄の E2 群では、甲状腺の腺内腔での発現は認められるものの、細胞質の染色性の亢進は明らかではなかった。血中の T4 の制御には、甲状腺からの産生と血中等での分解/排泄が関わっており、E2 投与では T4 の分解・排泄が抑制されている可能性が示唆された。

HE 染色標本では、雌雄とも AMT 群および PTU 群では甲状腺濾胞上皮細胞の肥大、過形成、単細胞壊死とうっ血所見が認められており（Figure 3）、TSH の亢進による影響が反映されていると考えられる。一方、E2 群では HE 染色標本上も、明らかな所見は認められておらず、組織像は甲状腺ホルモンレベルよりも甲状腺刺激ホルモンレベルに影響を強く受ける可能性が示唆された。

E. 結論

前年度に得られたサンプルにおいて、TSH 値の有意な変動が確認され、下垂体における抗 TSH 抗体を用いた免疫組織化学染色による評価に応用可能な検体が得られた。また、甲状腺における抗 T4 抗体を用いた免疫組織化学染色による検討では、血清での T4 低値を反映する結果が得られるとともに、変動の機序に関する情報も得られる可能性が示唆された。今後、他のホルモンの評価についても応用可能と考えられた。

F. 研究発表

F.1. 論文発表

なし

F.2.　学会発表

なし

G. 知的財産権の出願・登録状況

G.1. 特許取得

なし

G.2. 実用新案登録

なし

G.3.その他

なし

Table 1. Concentration of serum hormone in rats treated with various chemicals

		Basal	AMT	VD3	PTU	PB	AGT	E2
Male	TSH (ng/mL)	3.39 ± 1.29	23.92 ± 6.27 *	2.82 ± 1.18	14.92 ± 2.54	6.37 ± 2.38	10.89 ± 2.39	2.71 ± 0.98
		(5)	(5)	(5)	(5)	(5)	(5)	(5)
	T3 (ng/mL)	0.9 ± 0.1	0.4 ± 0.00 **	0.9 ± 0.2	0.4 ± 0.0 **	0.9 ± 0.0	0.7 ± 0.2	1.2 ± 0.1 **
	T4 (μg/mL)	4.5 ± 0.9	0.4 ± 0.0 **	4.1 ± 1.0	0.4 ± 0.0 **	4.1 ± 0.4	3.8 ± 2.1	6.4 ± 0.8 *
Female	TSH (ng/mL)	1.67 ± 0.75	42.54 ± 7.14 **	1.91 ± 0.67	35.89 ± 7.54 **	2.68 ± 1.01	24.25 ± 8.79	1.65 ± 0.31
		(5)	(4)	(3)	(5)	(5)	(5)	(5)
	T3 (ng/mL)	0.9 ± 0.2	0.4 ± 0.0 **	0.9 ± 0.1	0.4 ± 0.1 **	1.0 ± 0.1	0.7 ± 0.2 *	1.1 ± 0.1
	T4 (μg/mL)	3.2 ± 1.1	0.4 ± 0.0 **	4.0 ± 0.9	0.4 ± 0.0 **	2.9 ± 0.6	3.0 ± 1.5	4.5 ± 0.8
	Histologiy	np	Follicular cell hypertrophy Follicular cell hyperplasia Follilar cell single cell necrosis Congestion	np	Follicular cell hypertrophy Follicular cell hyperplasia Follilar cell single cell necrosis Congestion	np	Follicular cell hypertrophy	np

Values are expressed as mean ± standard deviation.
(); number of rat for TSH. Number of rat for T4/T3 is 5.
* and **; Significantly different from the basal diet group at p <0.05 and 0.01, respectively.

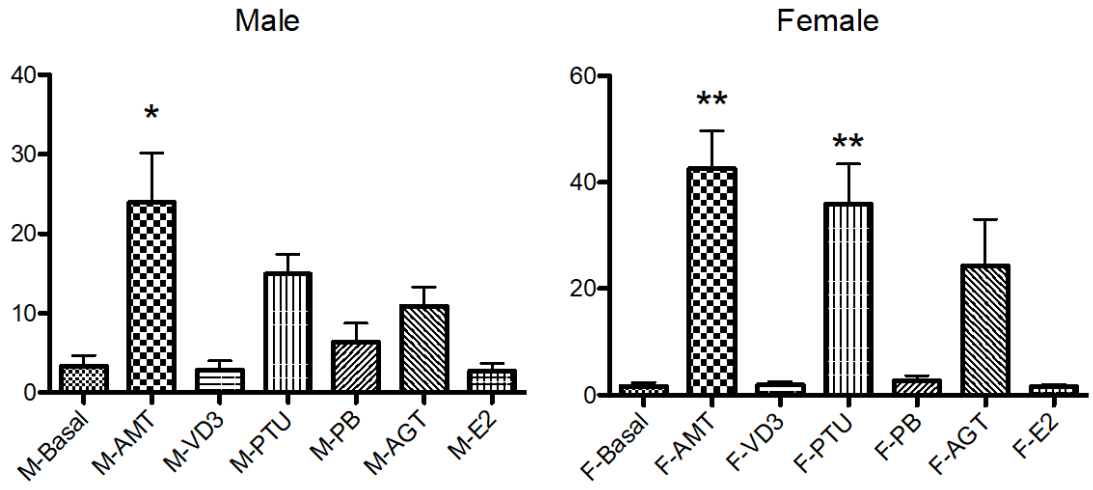

Figure 1 Concentration of serum thyroid stimulating hormone in rats

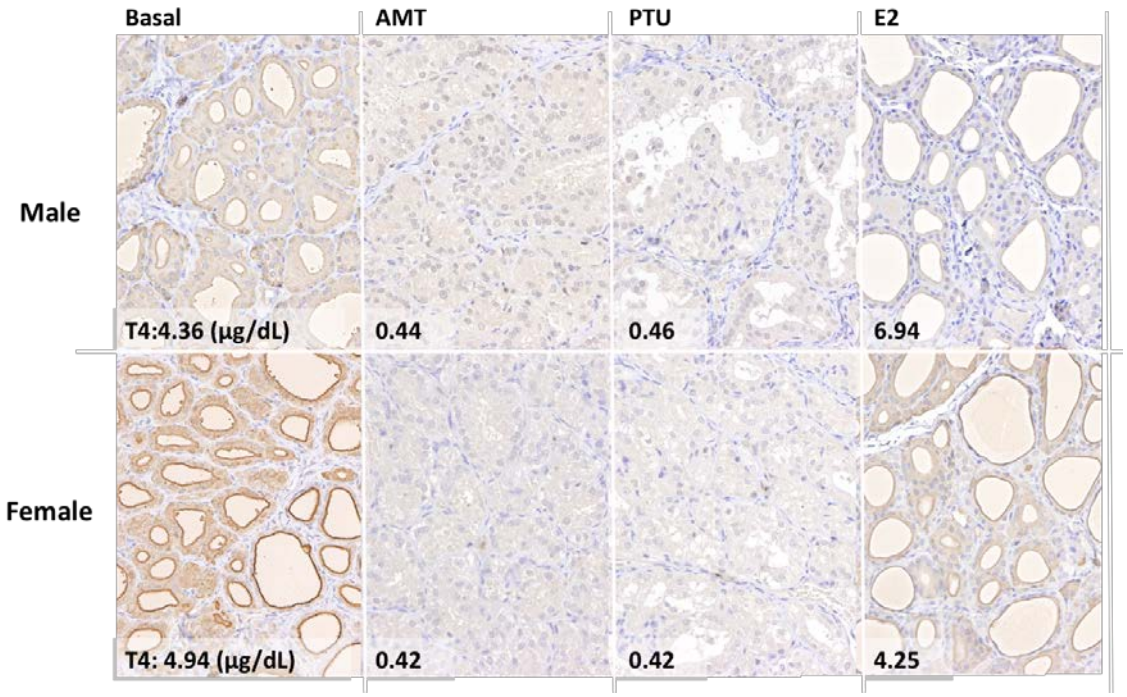

Figure 2 Representative observation of the immunohistochemical T4 expression in the thyroid of rat treated with various chemicals (numbers indicate serum T4 level)

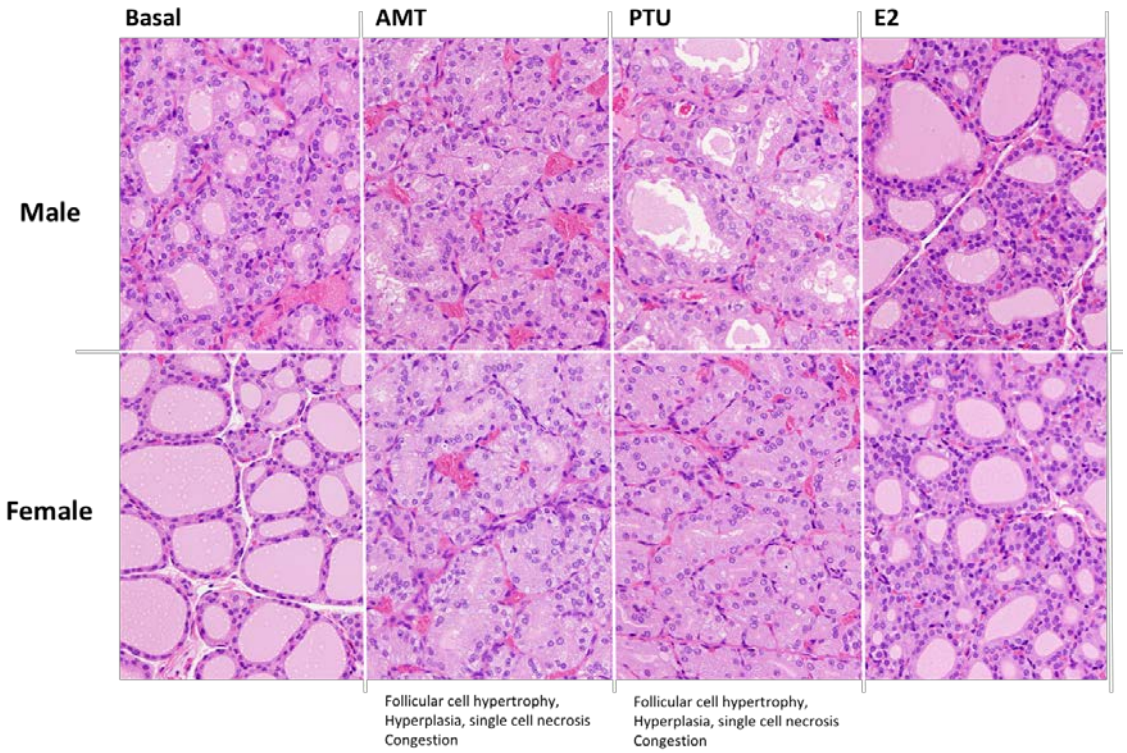

Figure 3 HE-staining in the thyroid of rat treated with various chemicals

厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）

OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

令和元年度 分担研究報告書

免疫毒性試験の TG および免疫毒性 AOP 開発

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研究要旨

現在、厚労科研（化学物質の動物個体レベルの免疫毒性データ集積とそれに基づくMulti-ImmunoTox assay (MITA)による予測性試験法の確立と国際標準化（H30-化学-一般-001））にて、MITAのOECDテストガイドライン化に向けてのvalidation試験を実施した。MITAのテストガイドライン化に際しては、その理論的根拠となる有害性発現経路（AOP: Adverse Outcome Pathway）の作成が不可欠である。テストガイドライン化を予定しているMITAの試験項目は、化学物質によるT細胞のIL-2転写抑制評価系と単球のIL-1転写抑制評価系である。前者に関しては、既に本厚労科研において足利らがInhibition of calcineurin activity leading to impaired T-cell dependent antibody response (AOP:154)を作成中であり、後者に関して、IL-1シグナルの欠損により易感染性を生じるというAOPを作成し9月に提出した。OECD EAGMSTの内部レビューアーの指摘を受け、タイトル、Molecular Initiating Event (MIE)の構成を変更し、またQuantitative Understanding, Empirical supportに関する論文を再調査し3月に再提出した。その結果、AOP : Inhibition of IL-1 binding to IL-1 receptor leading to increased susceptibility to infection (Aop:277)は外部評価へと移行した。

A. 研究目的

環境中に存在する何万という化学物質のなかには、免疫系を標的として健康被害を及ぼすものが多数存在する。したがって、免疫毒性は、消費者、生産者はもとより公衆衛生行政にとっても重要な課題となっている。現在、免疫毒性評価は動物実験を用いて行われているが、数万ともいわれる化学物質を網羅的に評価、管理するには、動物を用いない評価手法の開発が喫緊の課題である。その際、最終的には QSAR やカテゴリーアプローチ等の予測的評価法の開発が必須であるが、そのためにも免疫毒性 AOP の作成とそれに基づいた high throughput screening(HTP)法の確立が不可欠である。一方、我々はこれまでに多項目免疫毒性評価系 (MITA)を開発し、その data set の作成、有用性の検討、国際標準化へむけての validation 等を行ってきた。その中で、60 種類の化学物質を同じく我々が開発し OECD テストガイドラインに承認されている皮膚感作性試験 IL-8 Luc assay と MITA を組み合わせた modified mMITA により評価し、それらを複数のパラメータに関する効果発現

最低濃度 (Lowest observed effect level ; LOWEL)を基にクラスター分類することにより、免疫毒性物質が 6 種類のクラスターに分類できることを明らかにした。そこで、本課題では mMITA を多項目免疫毒性評価系として OECD テストガイドライン化することを目標に、その理論的背景となる adverse outcome pathway を作成する。

B. 研究方法

B.1. mMITA を評価系として用いる AOP の構築

我々がこれまでに開発した MITA は､T 細胞の IL-2、IFN-γ のプロモーター活性、単球の IL-1β､IL-8 プロモーター活性に与える化学物質の影響をルシフェラーゼ活性により high throughput に評価することができる（Kimura et al. Toxicol in Vitro, 2015）。さらに，これに IL-8 Luc assay を加えた mMITA では化学物質の皮膚感作性も評価できる。昨年度，人体への影響が明らかな免疫抑制剤を含む 60 種類の化学物質を評価した data set を作成し，MITA の 4 種類のパラメーターの内の２つと IL-8 Luc assay を用いて化学物質の免疫毒性による hierarchical clustering を施行したところ，化学物質が最大 6 つのクラスターに分けられることが明らかになった (Kimura et al. Arch Toxicol,2018)。これまでに化学物質の免疫毒性を clustering の手法で評価しようという試みの報告はない。現在，厚労科研 (化学物質の動物個体レベルの免疫毒性データ集積とそれに基づく Multi-ImmunoTox assay（MITA）による予測性試験法の確立と国際標準化（H30-化学-一般-001）)にて，MITA の OECD テストガイドライン化に向けての validation 試験を終了した。MITA のテストガイドライン化に際しては，その理論的根拠となる AOP の作成が不可欠である。ガイドライン化を予定している MITA の試験項目は，化学物質による T 細胞の IL-2 転写抑制評価系と単球の IL-1 転写抑制評価系である。本研究では特に後者に関して AOP を作成する。

B.2. AOP の国際的認証

完成した AOP は AOP-Wiki（https://aopwiki.org/wiki/index.php/Main_Page）にアップロードし、最終的には the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST)による承認を目指す。まず、各 AOP に関して、AOP Title, Authors, Abstract, Background, Summary of the AOP, Graphical Representation, Overall Assessment of the AOP, References の形式に沿って記載し AOP WIKI にアップロードする。

（倫理面への配慮）
特に必要とされない。

C. 研究結果

C.1. Inhibition of IL-1 signalingのAOP作成

本年度は，昨年度に引き続きIL-1シグナルの欠損により易感染性を生じるというAOPを作成した(Aop:277)。

C.2. AOP WIKI への登録

作成した AOP を Inhibition of IL-1 signaling という題名で 9 月に AOP-Wiki にアップロードした。(AOP：277)レビューアーより下記の指摘を受けこれらの修正を行い 3 月に再提出した。

- AOP のタイトルに Adverse Outcome を含めるようにとのコメントがありタイトルを Inhibition of IL-1 binding to IL-1

receptor leading to increased susceptibility to infection に変更した。

- abstract に weight of evidence などの記載も見られ冗長になっているとの指摘がありこれらの記載を他所に移し abstract を簡素化した。
- Molecular Initiating Event (MIE)は一つの AOP につき一つにするべきとの指摘があり MIE の構成を変更し MIE を一つとした。
- Key Events についても非専門家にも理解できるように記載するようにという指摘がありそのように修正した。
- Quantitative Understanding については適切な過去の論文が少ないことを記載し、関連を High から Moderate または Not Specified に変更した。

現時点での最新の AOP-Wiki への登録内容を Appendix 1 に示す。

その結果，AOP : Inhibition of IL-1 binding to IL-1 receptor leading to increased susceptibility to infection（Aop:277)は外部評価へと移行した。

D. 考察

他の厚労科研，化学物質の動物個体レベルの免疫毒性データ集積とそれに基づくMulti-ImmunoTox assay（MITA）による予測性試験法の確立と国際標準化（H30-化学-一般-001）にて，MITAのOECDテストガイドライン化に向けてのvalidation試験を終了した。申請に際して必要となるvalidation reportの作成において，MITA評価項目に関連するAOPの存在は不可欠である。ガイドライン化を予定しているMITAの試験項目は，化学物質によるT細胞のIL-2転写抑制，単球のIL-1転写抑制の評価系である。前者に関しては、既に本厚労科研において足利らがInhibition of calcineurin activity leading to impaired T-cell dependent antibody response (AOP: 154)を作成中であり，後者に関しては我々が作成中のAop:277)が対応する。

参照した過去の論文についてはノックアウトマウスを用いた実験や阻害剤を生体に投与した知見が中心であり Quantitative Understanding を裏付ける論文が少なく記載が困難であったが、レビューアーよりの提案もありその旨を記載し提出した。

empirical evidence については AOP に関する過去のいくつかの論文で表にまとめ提示しているが、それに倣い本 AOP でも表にまとめ提出した。

引用文献

1. Kimura, Y., Fujimura, C., Ito, Y., Takahashi, T., Terui, H., Aiba, S. Profiling the immunotoxicity of chemicals based on in vitro evaluation by a combination of the Multi-ImmunoTox assay and the IL-8 Luc assay. Arch Toxicol, 2018; 92. 2043-2054.
2. Kimura, Y., Fujimura, C., Ito, Y., Takahashi, T., Nakajima, Y., Ohmiya, Y., Aiba, S. Optimization of the IL-8 Luc assay as an in vitro test for skin sensitization. Toxicol In Vitro, 2015; 29. 1816-30.

E. 結論

AOP : Inhibition of IL-1 binding to IL-1 receptor leading to increased susceptibility to infection （Aop:277)を作成し，AOP-WIKIに登録した。その結果AOPは外部評価へと移行した。

F. 研究発表

F.1. 論文発表

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2. Kimura, Y., Yasuno, R., Watanabe, M., Kobayashi, M., Iwaki, T., Fujimura, C., Ohmiya, Y., Yamakage, K., Nakajima, Y., Kobayashi, M., Mashimo, N., Takagi, Y., Omori, T., Corsini, E., Germolec, D., Inoue, T., Rogen, E.L., Kojima, H., Aiba, S. An international validation study of the IL-2 Luc assay for evaluating the potential immunotoxic effects of chemicals on T cells and a proposal for reference data for immunotoxicchemicals. Toxicol In Vitro, 2020; in press.

F.2. 学会発表

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2. 木村 裕、安野理恵、渡美香、小林 美和子、岩城知子、藤村千鶴、近江谷克裕、山影康次、中島芳浩、真下奈々、高木佑実、大森 崇、小島 肇、相場節也 : Multi-ImmunoTox Assay (MITA)の予測性評価に必要な文献に基づく化学物質免疫毒性分類の試み 日本動物実験代替法学会第 32 回大会 つくば (2019.11)

G. 知的財産権の出願・登録状況

G.1. 特許取得

なし

AOP ID and Title:

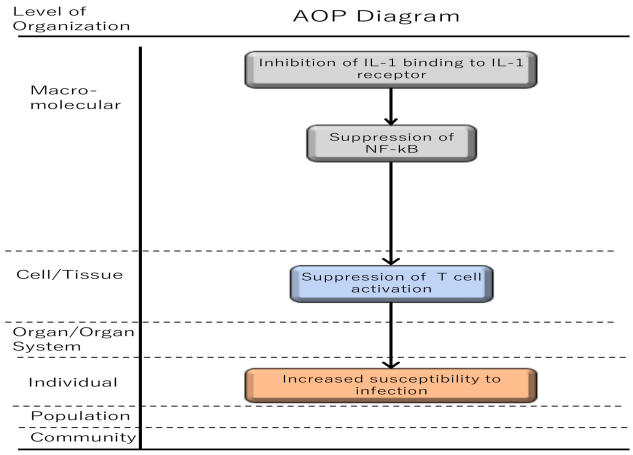
SNAPSHOT

Created at: 2020-03-31 06:49

AOP 277: Inhibition of IL-1 binding to IL-1 receptor leading to increased susceptibility to infection

Short Title: IL-1 inhibition

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Open for citation & comment	EAGMST Under Review	1.48	Included in OECD Work Plan

Abstract

The pleiotropic cytokine IL-1 mediates its biological functions via association with the signaling receptor IL-1R1. These may include initiation of innate immunity as well as acquired immunity, which are essential for assistance of host defense against infection. The trimeric complex consists of IL-1, IL-1R1 and IL-1R3 (a coreceptor, formerly IL-1R accessory protein) allows for the approximation of the Toll-IL-1-Receptor (TIR) domains of each receptor chain. MyD88 then binds to the TIR domains. The binding of MyD88 triggers a cascade of kinases that produce a strong pro-inflammatory signal leading to activation of NF-κB. The activation of NF-κB plays a principle role in the immunological function of IL-1. Namely, it stimulates innate immunity such as activation of dendritic cells and macrophages. It also stimulates T cells via activated dendritic function or directly. The activation of T cells is crucial for B cell proliferation and their antibody production. The cooperation by T cells and B cells constitutes a main part of host defense against infection.

In this AOP, we considered 2 MIEs, such as blocking IL-1 R and decreased IL-1 production. Either MIE leads to reduced IL-1 signaling. The biological plausibility of the signaling cascade from the activation of IL-1R to the activation of NF-kB is already confirmed. In addition, the biological plausibility that suppressed NF-kB activation leads to impaired T cell activation and antibody production lead to increased susceptibility to infection is supported by quite a few published works.

IL-1 also mediates several autoinflammatory syndromes. Therefore, several inhibitors against IL-1 signaling such as IL-1Ra (generic anakinra) , canakinumab (anti-IL-1β antibody) and rilonacept (soluble IL-1R) have been developed. After these inhibitors became available to treat these disorders, it became clear that these inhibitors increased the frequency of serious bacterial infection. Similarly, the experiments using knockout mice revealed that the lack of IL-1 signaling led to bacterial, tuberculosis or viral infection. These data suggest that chemicals as well as drugs can suppress IL-1 signaling through their inhibitory effects on IL-1β. Taken together, developing the AOP for inhibition of IL-1 signaling is mandatory.

Background

The pleiotropic cytokine IL-1 mediates its biological functions via association with the signaling receptor IL-1R1. These may include initiation of innate immunity and assistance of host defense against infection, and sometimes, mediation of autoinflammatory, such as cryopyrin-associated periodic syndrome, neonatal-onset multisystem inflammatory disease and familial Mediterranean fever. The trimeric complex consists of IL-1, IL-1R1 and IL-1R3 (a coreceptor, formerly IL-1R accessory protein) allows for the approximation of the Toll-IL-1-Receptor (TIR) domains of each receptor chain. MyD88 then binds to the TIR domains. The binding of MyD88 triggers a cascade of kinases that produce a strong pro-inflammatory signal leading to activation of NF-κB and fundamental inflammatory responses such as the induction of cyclooxygenase type 2, production of multiple cytokines and chemokines, increased expression of adhesion molecules, or synthesis of nitric oxide. (Dinarello, 2018) (Weber et al., 2010a, b).

IL-1 also mediates autoinflammatory, such as cryopyrin-associated periodic syndrome, neonatal-onset multisystem inflammatory disease and familial Mediterranean fever. Consequently, IL-1 family cytokines have sophisticated regulatory mechanisms to control their activities including proteolytic processing for their activation and the deployment of soluble receptors and receptor antagonists to limit their activities. Therefore, several inhibitors against IL-1 signaling have been developed. IL-1 receptor antagonist IL-1Ra was purified in 1990, and the cDNA was reported that same year. IL-1Ra binds IL-1R but does not initiate IL-1 signal transduction. (Dripps et al., 1991)Recombinant IL-1Ra (generic anakinra) is fully active in blocking the IL-1R1, and therefore, the activities of IL-1α and IL-1β. Anakinra was approved for the treatment of rheumatoid arthritis and cryopyrin-associated periodic syndrome (CAPS). Since its introduction in 2002 for the treatment of rheumatoid arthritis, anakinra has had a remarkable record of safety. However, Fleischmann et al. reported that serious infectious episodes were observed more frequently in the anakinra group (2.1% versus 0.4% in the placebo group) and other authors also reported the increased susceptibility to bacterial or tuberculosis infection (Genovese et al., 2004; Kullenberg et al., 2016; Lequerre et al., 2008; Migkos et al., 2015). As IL-1 signaling antagonists, two drugs went up to the market, canakinumab (anti-IL-1β antibody) and rilonacept (soluble IL-1R). Several reports described that the administration of these drugs led to increased susceptibility to infection. (De Benedetti et al., 2018; Imagawa et al., 2013; Lachmann et al., 2009; Schlesinger et al., 2012; Yokota et al., 2017). In addition to these human data, the experiments using knockout mice revealed that the lack of IL-1 signaling led to bacterial, tuberculosis or viral infection. (Guler et al., 2011; Horino et al., 2009; Juffermans et al., 2000; Tian et al., 2017; Yamada et al., 2000).

In this AOP, we considered inhibition of IL-1R activation as a MIE. The biological plausibility of the signaling cascade from the activation of IL-1R to the activation of NF-kB is already accepted. In addition, the biological plausibility that suppressed NF-kB activation leads to impaired T cell activation, resulting in impaired antibody production and impaired T cell and antibody production lead to increased susceptibility to infection is confirmed.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	1700	Inhibition of IL-1 binding to IL-1 receptor (https://aopwiki.org/events/1700)	Inhibition of IL-1 binding to IL-1 receptor
2	KE	202	Inhibition, Nuclear factor kappa B (NF-kB) (https://aopwiki.org/events/202)	Inhibition, Nuclear factor kappa B (NF-kB)
3	KE	1702	Suppression of T cell activation (https://aopwiki.org/events/1702)	Suppression of T cell activation
4	AO	986	Increase, Increased susceptibility to infection (https://aopwiki.org/events/986)	Increase, Increased susceptibility to infection

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition of IL-1 binding to IL-1 receptor (https://aopwiki.org/relationships/2002)	adjacent	Inhibition, Nuclear factor kappa B (NF-kB)	High	Moderate
Inhibition, Nuclear factor kappa B (NF-kB) (https://aopwiki.org/relationships/2003)	adjacent	Suppression of T cell activation	High	Moderate
Suppression of T cell activation (https://aopwiki.org/relationships/2004)	adjacent	Increase, Increased susceptibility to infection	High	Not Specified

Stressors

Name	Evidence
IL-1 receptor antagonist IL-1Ra (Anakinra)	High
anti-IL-1b antibody (Canakinumab)	High
soluble IL-1R (Rilonacept)	High
anti-IL-1b antibody (Gevokizumab)	High

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Sex Applicability

Sex	Evidence
Mixed	High

Although sex differences in immune responses are well known (Klein and Flanagan, 2016), there is no reports regarding the sex difference in IL-1 production, IL-1 function or susceptibility to infection as adverse effect of IL-1 blocking agent. Again, age-dependent difference in IL-1 signaling is not known.

The IL1B gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, and frog (https://www.ncbi.nlm.nih.gov/homologene/481 (https://www.ncbi.nlm.nih.gov/homologene/481)), and the Myd88 gene is conserved in human, chimpanzee, Rhesus monkey, dog, cow, rat, chicken, zebrafish, mosquito, and frog (https://www.ncbi.nlm.nih.gov/homologene?Db=homologene&Cmd=Retrieve&list_uids=1849 (https://www.ncbi.nlm.nih.gov/homologene?Db=homologene&Cmd=Retrieve&list_uids=1849)).

The NFKB1 gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, chicken, and frog.

275 organisms have orthologs with human gene NFKB1.

(https://www.ncbi.nlm.nih.gov/gene/4790 (https://www.ncbi.nlm.nih.gov/gene/4790))

The RELB gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, and frog.

216 organisms have orthologs with human gene RELB.

(https://www.ncbi.nlm.nih.gov/gene/5971 (https://www.ncbi.nlm.nih.gov/gene/5971))

These data suggest that the proposed AOP regarding inhibition of IL-1 signaling is not dependent on life stage, sex, age or species.

Essentiality of the Key Events

The experiments using knockout mice revealed that the deficiency of IL-1 signaling led to bacterial, tuberculosis or viral infection (Guler et al., 2011; Horino et al., 2009; Juffermans et al., 2000; Tian et al., 2017; Yamada et al., 2000).

IL-1 receptor antagonist IL-1Ra was purified in 1990, and the cDNA reported that same year. IL-1Ra binds IL-1R but does not initiate IL-1 signal transduction (Dripps et al., 1991). Recombinant IL-1Ra (generic anakinra) is fully active in blocking the IL-1R1, and therefore, the activities of IL-1α and IL-1β. Anakinra is approved for the treatment of rheumatoid arthritis and cryopyrin-associated periodic syndrome (CAPS). Since its introduction in 2002 for the treatment of rheumatoid arthritis, anakinra has had a remarkable record of safety. However, Fleischmann et al. (Fleischmann et al., 2003) reported that serious infectious episodes were observed more frequently in the anakinra group (2.1% versus 0.4% in the placebo group) and other authors reported the increased susceptibility to bacterial or tuberculosis infection (Genovese et al., 2004; Kullenberg et al., 2016; Lequerre et al., 2008; Migkos et al., 2015). Two IL-1 signaling antagonists, canakinumab (anti-IL-1b antibody) and rilonacept (soluble IL-1R) had been reported to increase susceptibility to infection (De Benedetti et al., 2018; Imagawa et al., 2013; Lachmann et al., 2009; Schlesinger et al., 2012).

In a similar way, defect of MyD88 signaling caused by knockout of mice gene or deficiency in human patient leads to the increased susceptibility to bacterial or tuberculosis infection (Fremond et al., 2004; Picard et al., 2010; Scanga et al., 2004; von Bernuth et al., 2008). Although MyD88 is also known to be involved in TLR signaling pathway, several reports suggested that MyD88-dependent response was IL-1 receptor-mediated but not TLR-mediated. These data suggest to essentiality of IL-1-MyD88 signaling pathway in host defense against infection.

Mice lacking NF-kB p50 are unable effectively to clear L. monocytogenes and are more susceptible to infection with S. peumoniae (Sha et al., 1995).

Weight of Evidence Summary

The recent review of IL-1 pathway by Weber et al. has clearly described the intracellular signaling event from the binding of IL-1a or IL-1b to IL-1R to the activation of NF-kB through the assemble of MyD88 to the trimelic complex composed of IL-1, IL-R1, and IL-1RacP. The sequentiality and essentiality of each signaling molecule have been demonstrated by mice lacking relevant molecules (Weber et al., 2010a, b).

There were several reports that described that administration of IL-1R antagonist or neutralizing antibody led to the suppression of downstream phenomena, which included internalization of IL-1 (Dripps et al., 1991), production of PGE_2 (Hannum et al., 1990; Seckinger et al., 1990b), IL-6 (Goh et al., 2014), and T cell proliferation (Seckinger et al., 1990a).

Biological plausibility

Inhibition of IL-1 binding to IL-1 receptor leads to Inhibition, Nuclear factor kappa B (NF-kB)

IL-1α and IL-1β independently bind the type I IL-1 receptor (IL-1R1), which is ubiquitously expressed. The IL-1R3 (formerly IL-1R accessory protein (IL-1RAcP)) serves as a co-receptor that is required for signal transduction of IL-1/IL-1RI complexes.

The initial step in IL-1 signal transduction is a ligand-induced conformational change in the first extracellular domain of the IL-1RI that facilitates recruitment of IL-1R3. the trimeric complex rapidly assembles two intracellular signaling proteins, myeloid differentiation primary response gene 88 (MYD88) and interleukin-1 receptor–activated protein kinase (IRAK) 4. This is paralleled by the (auto)phosphorylation of IRAK4, which subsequently phosphorylates IRAK1 and IRAK2, and then this is followed by the recruitment and oligomerization of tumor necrosis factor–associated factor (TRAF) 6. Activation of NF-κB by IL-1 requires the activation of inhibitor of nuclear factor B (IκB) kinase 2 (IKK2). Activated IKK phosphorylates IκBα, which promotes its K48-linked polyubiquitination and subsequent degradation by the proteasome. IκB destruction allows the release of p50 and p65 NF-κB subunits and their nuclear translocation, which is the central step in activation of NF-κB. Both NF-κBs bind to a conserved DNA motif that is found in numerous IL-1–responsive genes. (Weber et al., 2010a, b)

Inhibition, Nuclear factor kappa B (NF-kB) leads to Suppression of T cell activation

In T lineage cells, the temporal regulation of NF-kb controls the stepwise differentiation and antigen-dependent selection of conventional and specialized subsets of T cells in response to T cell receptor and costimulatory, cytokines and growth factor signals. Cytokines include cytokines produced from macrophage or monocyte such as IL-1b. (Gerondakis et al., 2014)

Suppression of T cell activation leads to Increase, Increased susceptibility to infection

First type immunity drives resistance to viruses and intracellular bacteria, such as Listeria monocytogenes, Salmonella spp. and Mycobacteria spp., as well as to intracellular protozoan parasites such as Leishmania spp. The T helper 1 signature cytokine interferon-γ has a central role in triggering cytotoxic mechanisms including macrophage polarization towards an antimicrobial response associated with the production of high levels of reactive oxygen species and reactive nitrogen species, activation of CD8 cytotoxic T lymphocytes and natural killer cells to kill infected cells via the perforin and/or granzyme B-dependent lytic pathway or via the ligation of surface death receptors; and B cell activation towards the production of cytolytic antibodies that target infected cells for complement and Fc receptor-mediated cellular cytotoxicity.

Resistance to extracellular metazoan parasites and other large parasites is mediated and/or involves second type immunity. Pathogen neutralization is achieved via different mechanisms controlled by T 2 signature cytokines, including interleukin-4, IL-5 and IL-13, and by additional type 2 cytokines such as thymic stromal lymphopoietin, IL-25 or IL-33, secreted by damaged cell. T 2 signature cytokines drive B cell activation towards the production of high-affinity pathogen-specific IgG1 and IgE antibodies that function via Fc-dependent mechanisms to trigger the activation of eosinophils, mast cells and basophils, expelling pathogens across epithelia.

T17 immunity confers resistance to extracellular bacteria such as Klebsiella pneumoniae, Escherichia coli, Citrobacter rodentium, Bordetella pertussis, Porphyromonas gingivalis and Streptococcus pneumoniae, and also to fungi such as Candida albicans, Coccidioides posadasii, Histoplasma capsulatum and Blastomyces dermatitidis. Activation of T 17 cells by cognate T cell receptor (TCR–MHC class II interactions and activation of group 3 innate lymphoid cells (ILC3s) via engagement of IL-1 receptor (IL-1R) by IL-1β secreted from damaged cells lead to the recruitment and activation of neutrophils. T 17 immunopathology is driven to a large extent by products of neutrophil activation, such as ROS and elastase (reviewed by Soares et al. (Soares et al., 2017).

Based on these evidences, the insufficient T cell or B cell function causes impaired resistance to infection.

Empirical support

This table summarizes the empirical support obtained from the experiment using several inhibitor or gene targeting mice.

concordance table empirical data						
				MIE	KE1	KE2
Reference	Chmical Initiator or deleted gene	dose	Species	Inhibition of IL-1 binding to IL-1 receptor	Inhibition, Nuclear factor kappa B (NF-kB)	Suppression of T cell activation
Dripps et al. 1991	IL-1Ra (anakinra)			Equilibrium binding and kinetic experiments show that IL-1ra binds to the 80-kDa IL-1 receptor on the murine thymomcae II line EL4 with an affinity (K_D = 150 pM) approximately equal to that of IL-la and IL-1b for this receptor		
Sigma-Aldrich Specification Sheet	IL-1Ra (anakinra)			Determined by its ability to inhibit the IL-1alpha stimulation of murine D10S cell. The expected ED50 is 20-40 ng/ml in the presence of 50 pg/ml of IL-1alpha.		
Fleischmann et al. 2003	IL-1Ra (anakinra)	100 mg of anakinra or placebo, administered daily by subcutaneous injection	human			
Genovese et al. 2004	IL-1Ra (anakinra)	treated with subcutaneous etanercept only (25 mg twice weekly), full-dosage etanercept (25 mg twice weekly) plus anakinra (100 mg/day), or half-dosage etanercept (25 mg once weekly) plus anakinra (100 mg/day) for 6 months	human			
Kullenberg et al. 2016	IL-1Ra (anakinra)	administered as daily s.c. injections	human			
Lequerre et al. 2008	IL-1Ra (anakinra)	treated with anakinra (1–2 mg/kg/day in children, 100 mg/day in adults)	human			
Migkos et al. 2015	IL-1Ra (anakinra)		human			
Settas et al. 2007	IL-1Ra (anakinra)		human			
Lee et al. 2004	IL-1Ra (anakinra)		intrathecal administration of IL-1ra (6 mg)		intrathecal pretreatment with IL-1ra (6 mg) or YVAD (0.5 mg) significantly inhibited NF-kB DNA-binding activity upregulation bilaterally (Fig. 3C). The intrathecal administration of IL-1ra or YVAD into non-inflamed animals produced no significant change in the DNA-binding activity of NF-kB p65.	
Vallejo et al. 2014	IL-1Ra (anakinra)	In diabetic rats treated with anakinra (100 or 160 mg/Kg/day for 3 or 7 days before sacrifice)	rat		In diabetic rats treated with anakinra (100 or 160 mg/Kg/day for 3 or 7 days before sacrifice) a partial improvement of diabetic endothelial dysfunction occurred, together with a reduction of vascular NADPH oxidase and NF-κB activation.	
Dhimolea et al. 2010	canakinumab			Canakinumab binds to human IL-1β with high affinity; the antibody-antigen dissociation equilibrium constant is approximately 35–40 pM. Cmax was 1.2, 1.2 and 1.5 pM for 1, 3 and 10 mg/kg antibody respectively, at days 42–56 after the first infusion.		
De Benedetti et al. 2018	canakinumab	150 mg subcutaneously every 4 weeks	human			
Imagawa et al. 2013	canakinumab	either 150 mg s.c. or 2 mg/kg for patients with a body weight ≤ 40 kg every 8 weeks for 24 weeks received	human			
Lachmann et al. 2009	canakinumab	150 mg of canakinumab subcutaneously every 8 weeks for up to 24 weeks	human			

Schlesinger et al. 2012	canakinumab	one dose of canakinumab 150 mg	human			
Textbook of Pediatric Rheumatologyrilonacept (Sixth Edition), 2011			human	Rilonacept has a very high binding affinity for IL-1 (dissociation constant ~1 pM), and it is specific for IL-1β and IL-1α.		
Hoffman et al. 2008	rilonacept	weekly subcutaneous injections (160 mg)	human			
Roell et al. 2010	gevokizumab (XOMA 052)		human		XOMA 052 neutralizes IL-1b stimulation of NFkB activation in HeLa cells stably expressing an NFkB-luciferase reporter construct with an IC_{50} of ~1 pM at the EC_{50} for this assay (25 pg/ml IL-1b).	
Mansouri et al. 2015	gevokizumab (XOMA 052)	receive gevokizumab 60 mg subcutaneously every 4 weeks for a total of three injections (12 weeks) with a 4-week follow-up period	human			
Issafras et al. 2014	gevokizumab (XOMA 052)		human (HeLa cells stably transfected with a nuclear factor-kB (NF-kB) luciferase reporter plasmid)		an average K_B value (mean±S.D., n=3) of 4.8±4.4 pM	
Palombella et al. 1994	MG-132		human (in vitro)		Both MG115 and MG132 (at 20-40 mM) markedly inhibited the formation of p50 in HeLa S100 extracts (Figure 4A, lanes 8-13).	
Hellerbrand et al. 1998	MG-132		rat (in vitro)		ALLN (Fig. 3A) and MG132 (Fig. 3B) (10 mg/mL = 21mM) reduced the cytokine-mediated NFkB activation.	
Arlt et al. 2001	MG-132		human (in vitro)		In all cell lines, gliotoxin, MG132 (10 mM) or sulfasalazine strongly reduced VP16-induced NF-kB-driven luciferase expression.	
Ortiz-Lazareno et al. 2008	MG-132		human (in vitro)		The increase in NF-kB activation induced by LPS+PMA diminished significantly from 3.27-fold to 0.94-fold in the group treated with MG132(10 mM) and later stimulated with LPS+PMA ($P < 0.002$). The activation of NF-kB induced by LPS+PMA was blocked by MG132.	
Yu and Malek 2001	MG-132		mice (in vitro)			MG132 (50mM) stabilized IL-2- phosphorylated STAT5, which
Wang et al. 2011	MG-132		human (in vitro)			after 2 h in culture (Fig. 5A, lan CMV-specific cytotoxicity of CD decreased in the presence of M
Ohkusu-Tsukada et al. 2018	MG-132	repeatedly i.p. injected 200 nmol of MG132 on days 0, 3, 5, 7, 9, 11, 13, 15, 17, and 19.	mice (in vivo)			In vivo MG132 administration t DNFB-induced dermatitis reduc maintained the level of Th1 cel alleviation of dermatitis lesions serum IgE hyperproduction an
Satou et al. 2004	bortezomib		human (in vitro, in vivo)			potently inhibits the growth of a cells both in vivo and in vitro
Orciuolo et al. 2007	bortezomib	0.1 mM, 1 mM, 10 mM	human (in vitro)			the percentage of CD69/TNFa with the increment of bortezom
Matsumoto et al. 2005	dehydroxymethylepoxyquinomicin (DHMEQ)		human		The addition of DHMEQ (10 mg/mL) completely inhibited the activated NF-KB for at least 8 hours.	
Nishioka et al. 2008	dehydroxymethylepoxyquinomicin (DHMEQ)		human (in vitro)		DHMEQ (1mg/mL) blocked PHA-induced nuclear translocation of NF-kB in Jurkat cells via inhibition of degradation of IkBa.	Exposure of PBMC to PHA grea expression of IFN-g, IL-2 and TNF-a (Fig. these cells with DHMEQ (1 mg/ reduced PHA-stimulated expression of t (Fig. 3a). Similarly, PHA increa and IFN-c in Jurkat cells and pr cells with DHMEQ (1 mg/ml) de by approximately half (Fig. 3b).
Alessiani et al. 1991	FK 506		human			Five of eight deaths were due t Overall, 50% of patients develo 38% suffered severe ones.
Fung et al. 1991	FK 506		human			The incidence of serious infecti of FK 506, has not appeared to incidence of serious infections seen in a historical group of pa is that the incidence of cytomeg not appear to be increased wh patients on CyA.
Ekberg et al. 2007	cyclosporine		human			The most commonly reported s were cytomegalovirus (CMV) v infection and lymphocele (Tabl patients with opportunistic infec serious) was also similar amon cytomegalovirus infection was t opportunistic infection (Table 3
Guler et al. 2011	i) IL-1RI$^{-/-}$ ii) Autologous Qb virus-like particle-based vaccines against IL-1a and IL-1b	ii) immunized s.c. three times before (at week: –5, –3 and –1) and once at week 10 post-infection	mice			
Parnet et al. 2003	IL-1RI$^{-/-}$				Activation of NFkB in response to IL-1b was no longer apparent in IL-1RI knockout mice, confirming that this receptor is essential for the transduction of IL-1 signal in the pituitary,	

Yamada et al. 2001	NF-kB $p50^{-/-}$	knockout mice	mice	
Weih et al. 1995	$RelB^{-/-}$	knockout mice	mice	RelB-deficient animals also hac immunity, as observed in conta experiments.
Lin et al. 2015	Secreted IL-1α expression		mice	Both the percent and number c CD8+ T cells, and CD69+ CD4- the expression of secreted IL-1 IL-1b, but not IL-1a, is required T cell activation and the inducti inflammation in the delayed-typ responses
Horai et al. 2006	IL-1α-/-, IL-1β-/-, IL-1αβ-/-	knockout mice	mice	

Considerations for Potential Applications of the AOP (optional)

The impaired IL-1 signaling can lead to decreased host resistance to various infections. Therefore, the test guideline to detect chemicals that decrease IL-1 signaling is required to support regulatory decision-making. This AOP can promote the understanding of the usefulness of the test guideline.

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Appendix 1

List of MIEs in this AOP

Event: 1700: Inhibition of IL-1 binding to IL-1 receptor (https://aopwiki.org/events/1700)

Short Name: Inhibition of IL-1 binding to IL-1 receptor

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:277 - Inhibition of IL-1 binding to IL-1 receptor leading to increased susceptibility to infection (https://aopwiki.org/aops/277)	MolecularInitiatingEvent

Stressors

Name
IL-1 receptor antagonist IL-1Ra (Anakinra)
anti-IL-1b antibody (Canakinumab)
soluble IL-1R (Rilonacept)

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
macrophage

Organ term

Organ term
immune system

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

IL-1 is known to mediates autoinflammatory syndrome, such as cryopyrin-associated periodic syndrome, neonatal-onset multisystem inflammatory disease and familial Mediterranean fever. The stressors of this MIE, such as anakinra, canakinumab, and rilonacept have been already used to treat these autoinflammatory syndrome associated with overactivation of IL-1 signaling (Quartier, 2011).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Although sex differences in immune responses are well known (Klein and Flanagan, 2016), there is no reports regarding the sex difference in IL-1 production, IL-1 function or susceptibility to infection as adverse effect of IL-1 blocking agent. Again, age-dependent difference in IL-1 signaling is not known.

The IL1B gene is conserved in chimpanzee, rhesus monkey, dog, cow, mouse, rat, and frog (https://www.ncbi.nlm.nih.gov/homologene/481 (https://www.ncbi.nlm.nih.gov/homologene/481)), and the Myd88 gene is conserved in human, chimpanzee, rhesus monkey, dog, cow, rat, chicken, zebrafish, mosquito, and frog (https://www.ncbi.nlm.nih.gov/homologene?Db=homologene&Cmd=Retrieve&list_uids=1849 (https://www.ncbi.nlm.nih.gov/homologene?Db=homologene&Cmd=Retrieve&list_uids=1849)).

These data suggest that the proposed AOP regarding inhibition of IL-1 signaling is not dependent on life stage, sex, age or species.

Key Event Description

IL-1α and IL-1β independently bind the type I IL-1 receptor (IL-1R1), which is ubiquitously expressed. IL-1Ra binds IL-1R but does not initiate IL-1 signal transduction (Dripps et al., 1991). Recombinant IL-1Ra (anakinra) is fully active in blocking the IL-1R1, and therefore, the biological activities of IL-1α and IL-1β. The binding of IL-1α and IL-1β to IL-1R1 can be suppressed by soluble IL-1R like rilonacept (Kapur and Bonk, 2009). The binding of IL-1β to IL-1R1 can be inhibited by anti-IL-1β antibody (anti-IL-1β antibody) (Church and McDermott, 2009).

How it is Measured or Detected

1. Competitive inhibition binding experiments using ^{125}I-IL-1a to type I IL-1R present on EL4 thymoma cells, 3T3 fibroblasts, hepatocytes, and Chinese hamster ovary cells expressing recombinant mouse type I IL-1R (McIntyre et al., 1991; Shuck et al., 1991).
2. Measure the ability of the reagent to neutralize the bioactivity of human IL-1β on primary human fibroblasts in vitro(Alten et al., 2008)

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List of Key Events in the AOP

Event: 202: Inhibition, Nuclear factor kappa B (NF-kB) (https://aopwiki.org/events/202)

Short Name: Inhibition, Nuclear factor kappa B (NF-kB)

Key Event Component

Process	Object	Action
I-kappaB kinase/NF-kappaB signaling	transcription factor NF-kappa-B subunit	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:14 - Glucocorticoid Receptor Activation Leading to Increased Disease Susceptibility (https://aopwiki.org/aops/14)	KeyEvent
Aop:278 - IKK complex inhibition leading to liver injury (https://aopwiki.org/aops/278)	KeyEvent
Aop:277 - Inhibition of IL-1 binding to IL-1 receptor leading to increased susceptibility to infection (https://aopwiki.org/aops/277)	KeyEvent

Stressors

Name
IL-1 receptor antagonist IL-1Ra (Anakinra)
anti-IL-1b antibody (Canakinumab)
soluble IL-1R (Rilonacept)

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
macrophage

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The binding of sex steroids to their respective steroid receptors directly influences NF-κB signaling, resulting in differential production of cytokines and chemokines (McKay and Cidlowski, 1999; Pernis, 2007). 17b-estradiol regulates pro-inflammatory responses that are transcriptionally mediated by NF-κB through a negative feedback and/or transrepressive interaction with NF-κB (Straub, 2007). Progesterone suppresses innate immune responses and NF-κB signal transduction reviewed by Klein et al. (Klein and Flanagan, 2016). Androgen-receptor signaling antagonises transcriptional factors NF-κB(McKay and Cidlowski, 1999).

Key Event Description

The NF-kB pathway consists of a series of events where the transcription factors of the NF-kB family play the key role. The NF-κB pathway can be activated by a range of stimuli, including TNF receptor activation by TNF-a, or IL-1R1 activation by IL-1a or b. Upon pathway activation, the IKK complex will be phosphorylated, which in turn phosphorylates IkBa. This NF-kB inhibitor will be K48-linked ubiquitinated and degradated, allowing NF-kB to translocate to the nucleus. There, this transcription factor can express pro-inflammatory and anti-apoptotic genes. Furthermore, negative feedback genes are also transcribed and include IkBa and A20. When the NF-kB pathway is inhibited, its translocation will be delayed (or absent), resulting in less or no regulation of NF-kB target genes. This can be achieved by IKK inhibitors, proteasome inhibitors, nuclear translocation inhibitors or DNA-binding inhibitors. (Frederiksson 2012)(Gupta et al. 2010)(Huppelschoten 2017)(Liu et al. 2017). Therefore, inhibition of IL-1R1 activation suppresses activation of NF-kB.

How it is Measured or Detected

NF-kB transcriptional activity: Beta lactamase reporter gene assay (Miller et al. 2010). NF-kB transcription: Lentiviral NF-kB GFP reporter with flow cytometry (Moujalled et al. 2012)

NF-κB translocation: RelA-GFP reporter assay (Frederiksson 2012) (Huppelschoten 2017)

IκBa phosphorylation: Western blotting (Miller et al. 2010)

NF-κB p65 (Total/Phospho) ELISA

ELISA for IL-6, IL-8, and Cox

References

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Straub, R.H., 2007. The complex role of estrogens in inflammation. Endocr Rev 28, 521-574.

Event: 1702: Suppression of T cell activation (https://aopwiki.org/events/1702)

Short Name: Suppression of T cell activation

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:277 - Inhibition of IL-1 binding to IL-1 receptor leading to increased susceptibility to infection (https://aopwiki.org/aops/277)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
T cell

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Description

T cells are key orchestrators of the response against pathogens and are also fundamental in maintaining self-tolerance. A number of clinically important conditions have been described in which T-cell functions are altered, as in AIDS or upon immunosuppression after application of various immunosuppressive drugs to treat autoimmune disorders or allogeneic graft rejection. T-cell progenitors differentiate in the thymus into immature T cells that acquire the expression of the T-cell receptor (TCR), which recognizes antigen peptides from pathogens presented along with major histocompatibility complex (MHC). In addition to the TCR, T cells are characterized by expression of the co-receptor molecules CD4 and CD8 on their cell surface. CD4+ T cells, also called T helper (Th) cells, recognize antigen/MHC-II complexes on antigen presenting cells (APCs) and coordinate the activation of other immune cells including B cells, macrophages, etc.

Therefore, CD4+ T cells are crucial for coordination of the immune response and for the elimination of invading pathogens. On the other hand, CD8+ T cells, referred to as T cytotoxic cells, recognize antigen/MHC-I complexes and are responsible for the killing of pathogen-infected cells.

T-cell activation and differentiation depends on antigen presenting cells (APCs) such as dendritic cells (DCs), macrophages and B cells. depending on the insult affecting a given tissue. Different subsets of DCs can be generated that in turn are able to coordinate the differentiation of a particular Th subset. To date, the following Th subsets have been described: Th1, Th2, Th9, Th17, Th22, Tfh (follicular helper T cells), Tr1 (type 1regulatory T cells) and Treg (regulatory T cells), each possessing a specific function in the elimination of pathogens. (reviewed by Simeoni et al. (Simeoni et al., 2016))

Although CD4 T cells are able to commit to Th1, Th2 and Th17 lineages in the absence of IL-1R signaling at steady state, these committed CD4 T cells are unable to effectively secrete their cytokines upon TCR ligation. Namely, IL-1 is indispensable for CD4 T cell effector function. (Lin et al, 2015)

Moreover, since full activation of B cells and antibody production and class switch depends on T cell help. The impaired activation of T cells leads to impaired B cell activation and antibody production (reviewed by Mok (Mok, 2010)).

How it is Measured or Detected

T cell activation can be evaluated by measuring IL-2 production by ELISA or T cell proliferation by incorporation of the analysis of CFSE labeled T cells or [^{3}H]thymidine incorporation.

References

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Simeoni, L., Thurm, C., Kritikos, A., et al., 2016. Redox homeostasis, T cells and kidney diseases: three faces in the dark. Clin Kidney J 9, 1-10.

List of Adverse Outcomes in this AOP

Event: 986: Increase, Increased susceptibility to infection (https://aopwiki.org/events/986)

Short Name: Increase, Increased susceptibility to infection

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:277 - Inhibition of IL-1 binding to IL-1 receptor leading to increased susceptibility to infection (https://aopwiki.org/aops/277)	AdverseOutcome

Stressors

Name
IL-1 receptor antagonist IL-1Ra (Anakinra)
anti-IL-1b antibody (Canakinumab)
soluble IL-1R (Rilonacept)

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The increased susceptibility to infection caused by IL-1RA or anti-IL-1 antibody has been reported in both humans and mice. (Fleischmann et al., 2003; De Benedetti et al., 2018; Hirsch et al., 1996)

Key Event Description

The protection of host against microbial infection depends on both innate and acquired immunity. In particular, both T cell and antibody production by B cells play a principal role.

How it is Measured or Detected

By comparison of the incidence of infection between individuals exposed to stressors and non-exposed individuals.

Regulatory Significance of the AO

After L-1R antagonist or neutralizing antibody such as IL-1Ra (generic anakinra), canakinumab (anti-IL-1b antibody) and rilonacept (soluble IL-1R) became available to treat some of autoinflammatory syndromes, it became clear that these inhibitors increased the frequency of serious bacterial infection (De Benedetti et al., 2018; Genovese et al., 2004; Imagawa et al., 2013; Kullenberg et al., 2016; Lachmann et al., 2009; Lequerre et al., 2008; Migkos et al., 2015; Schlesinger et al., 2012; Yokota et al., 2017).

References

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2002: Inhibition of IL-1 binding to IL-1 receptor leads to Inhibition, Nuclear factor kappa B (NF-kB) (https://aopwiki.org/relationships/2002)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of IL-1 binding to IL-1 receptor leading to increased susceptibility to infection (https://aopwiki.org/aops/277)	**adjacent**	**High**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

The initial step in IL-1 signal transduction is a ligand-induced conformational change in the first extracellular domain of the IL-1RI that facilitates recruitment of IL-1RacP. Through conserved cytosolic regions called Toll- and IL-1R–like (TIR) domains, the trimeric complex rapidly assembles two intracellular signaling proteins, myeloid differentiation primary response gene 88 (MYD88) and interleukin-1 receptor–activated protein kinase (IRAK) 4. IL-1, IL-1RI, IL-RAcP, MYD88, and IRAK4 form a stable IL-1–induced first signaling module. The binding of MyD88 triggers a cascade of kinases that produce a strong pro-inflammatory signal leading to activation of NF-κB. reviewed by Brikos et al. (Brikos et al., 2007) and Weber et al. (Weber et al., 2010).

Therefore, the suppression of the binding of IL-1 to IL-1R1 suppresses activation of NF-κB.

Evidence Supporting this KER

Biological Plausibility

IL-1α and IL-1β independently bind the type I IL-1 receptor (IL-1R1), which is ubiquitously expressed. IL-1Ra binds IL-1R but does not initiate IL-1 signal transduction (Dripps et al., 1991). Recombinant IL-1Ra (anakinra) is fully active in blocking the IL-1R1, and therefore, the biological activities of IL-1α and IL-1β. The binding of IL-1α and IL-1β to IL-1R1 can be suppressed by soluble IL-R like rilonacept. The binding of IL-1β to IL-1R1 can also be inhibited by anti-IL-1β antibody (anti-IL-1β antibody). Therefore, the inhibition of IL-1 binding to IL-1R1 cannot activate NF-κB.

Empirical Evidence

IL-1Ra blocks IL-1 signaling:

IL-1Ra down-modulates EGF receptor (3 nM of ED50) by IL-1 stimulation (Dripps et al., 1991)

IL-1Ra suppresses IL-1-induced endothelial cell-leukocyte adhesion (approximately 10 ng/ml of ED50) (Dripps et al., 1991)

IL-1Ra suppresses rhIL-1a-induced mouse thymocytes proliferation (ED50 almost 3 mg/mL) (Arend et al., 1990)

IL-1Ra competed for binding of ^{125}I-IL-1a to type I IL-1R present on EL4 thymoma cells, 3T3 fibroblasts, hepatocytes, and Chinese hamster ovary cells expressing recombinant mouse type I IL-1R. The IC50 values for IL-1ra binding (ranging from 2 to 4 ng/ml) were similar to those of IL-1a. (McIntyre et al., 1991)

Recombinant mIL-1Ra competitively inhibited ^{125}I-labeled IL-1 alpha binding to murine type I IL-1R present on EL4 6.1 cells (Ki value of 0.21 nM) and antagonized IL-1-stimulated co-mitogenesis in murine thymocytes (0.7 x 10(6)-1.1 x 10(6) units/mg). (Shuck et al., 1991)

Peripheral blood mononuclear cells (PBMC) obtained after completion of the IL-Ira infusion synthesized significantly less interleukin 6 ex vivo than PBMC from saline-injected controls. (Granowitz et al., 1992)

Canakinumab (ACZ885, Ilaris) blocks IL-1 signaling

Canakinumab binds to human IL-1β with high affinity; the antibody-antigen dissociation equilibrium constant is approximately 35–40 pM(Dhimolea, 2010).

The antibody binds to human IL-1β with high affinity (about 40 pmol/l). The antibody was found to neutralize the bioactivity of human IL-1β on primary human fibroblasts in vitro 44.6 pmol/l (7.1 ± 0.56 ng/ml; n = 6) of ED50. Application of Canakinumab intraperitoneally 2 hours before injecting the IL-1β producing cells completely suppressed joint swelling (0.06 mg/kg of EC50) (Alten et al., 2008).

Primary human fibroblasts are stimulated with recombinant IL-1b or conditioned medium obtained from LPS-stimulated human PBMCs in the presence of various concentrations of Cankinumab or IL-1RA ranging from 6 to 18,000 pM. Supernatant is taken after 16 h stimulation and assayed for IL-6 by ELISA. Canakinumab typically have 1 nM or less of EC50 for inhibition of IL-6 production (Canakinumab Patent Application WO02/16436.)

Rilonacept (IL-1 Trap, Arcalyst) blocks IL-1 signaling:

Incubation of the human MRC5 fibroblastic cell line with IL-1β induces secretion of IL-6. At a constant amount of IL-1β (4 pM), the IC50 of the IL-1 trap is ~2 pM. Another unique property of the IL-1 trap is that it not only blocks IL-1β, but also blocks IL-1α with high affinity (KD = ~3 pM; data not shown). The titration curve of IL-1 trap in the presence of 10 pM IL-1β shows an IC50 of 6.5 pM, which corresponds to a calculated KD of 1.5 pM (This affinity is 100 times higher than that of the soluble single component receptor IL-1RI (Economides et al., 2003).

Quantitative Understanding of the Linkage

See Empirical Evidence.

Response-response relationship
IL-1Ra blocks IL-1 signaling:

Suppression of IL-1-induced IL-1, TNFa, or IL-6 synthesis was dose-dependent (P ≦ .0001). At a twofold molar excess, IL-Ira inhibited IL-1-induced IL-1 or TNFa synthesis by 50% (P < .01); an equimolar concentration of IL-Ira inhibited synthesis of these two cytokines by over 20% (P < .05). A 10-fold molar excess of IL-Ira over IL-Ib reduced IL-Ib-induced IL-Ia by 95% (P = .01) and IL-Ia-induced IL-1b by 73% (P < .01). In elutriated monocytes, a 10-fold molar excess of IL-Ira reduced IL-Ib-induced IL-Ia by 82% (P < .05), TNFa by 64% (P = .05), and IL-6 by 47% (P < .05). (Granowitz et al., 1992)

Rilonacept (IL-1 Trap, Arcalyst) blocks IL-1 signaling:

The titration curve of IL-1 trap in the presence of 10 pM IL-1β shows an IC50 of 6.5 pM, which corresponds to a calculated KD of 1.5 pM (This affinity is 100 times higher than that of the soluble single component receptor IL-1RI (Economides et al., 2003).

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Schlesinger, N., Alten, R.E., Bardin, T., et al., 2012. Canakinumab for acute gouty arthritis in patients with limited treatment options: results from two randomised, multicentre, active-controlled, double-blind trials and their initial extensions. Ann Rheum Dis 71, 1839-1848.

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Relationship: 2003: Inhibition, Nuclear factor kappa B (NF-kB) leads to Suppression of T cell activation (https://aopwiki.org/relationships/2003)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of IL-1 binding to IL-1 receptor leading to increased susceptibility to infection (https://aopwiki.org/aops/277)	**adjacent**	**High**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

In T cells, NF-kB can be activated by several pathways of signal transduction. The engagement of the TCR by major histocompatibility complex (MHC) plus antigen initiates downstream CD3 immunotyrosine activation motif (ITAM) phosphorylation by the Src family kinases, FYN and leukocyte C-terminal src kinase (LCK). Phosphorylated CD3 activates the T cell specific tyrosine kinase, zeta-chain associated protein kinase (ZAP-70), which ultimately trigger calcium release and protein kinase (PK)C activation, respectively. Activation of a specific PKC isoform, PKC*u*, connects the above described TCR proximal signaling events to distal events that ultimately lead to NF-*k*B activation. Importantly, PKC*u* activation is also driven by engagement of the T cell co-stimulatory receptor CD28 by B7 ligands on antigen presenting cells (APCs). In addition, the stimulation of T cells by IL-1 activates NF-*k*B as already described before. Once in the nucleus, NF-*k*B governs the transcription of numerous genes involved in T cell survival, proliferation, and effector functions (Paul and Schaefer, 2013).

Evidence Supporting this KER

Biological Plausibility

Although CD4 T cells are able to commit to Th1, Th2 and Th17 lineages in the absence of IL-1R signaling at steady state, these committed CD4 T cells are unable to effectively secrete their cytokines upon TCR ligation. Namely, IL-1 is indispensable for CD4 T cell effector function. (Lin et al, 2015)

RelB deficient mice had an impaired cellular immunity, as observed in contact sensitivity reaction (Weih et al., 1995).

Delayed-type hypersensitivity (DTH) responses were significantly suppressed in IL-1b-deficient and IL-1a/b-deficient mice. Lymph node cells derived from antigen-sensitized IL-1b-deficient and IL-1a/b-deficient mice and IL-1R type I-deficient mice, exhibited reduced proliferative responses against antigen. (Nambu et al., 2006).

Empirical Evidence
RelB deficient mice had an impaired cellular immunity, as observed in contact sensitivity reaction (Weih et al., 1995).

Quite a few NF-kB inhibitors have been reported. MG132, bortezomib, curcumin, DHMEQ(Dehydroxymethylepoxyquinomicin), naringin, sorafenib, genistein and parthenolide are some of representatives (Pordanjani and Hosseinimehr, 2016).

Interferon-γ (IFN-γ) production in response to CMV-infected fibroblasts was reduced under the influence of MG132 in a dose-dependent manner. A marked reduction was observed at 0.5 µM. Likewise, CMV-specific cytotoxicity of CD8(+) T cells was decreased in the presence of MG132 (Wang et al., 2011).

In vivo MG132 administration to NC/Nga mice with DNFB-induced dermatitis reduced Th17 cells but maintained the level of Th1 cells, resulting in the alleviation of dermatitis lesions by decreasing both serum IgE hyperproduction and mast cell migration (Ohkusu-Tsukada et al., 2018).

Proteasome inhibitor, bortezomib, potently inhibits the growth of adult T-cell leukemia cells both in vivo and in vitro (Satou et al., 2004). Bortezomib inhibits T-cell function versus infective antigenic stimuli in a dose-dependent manner in vitro (Orciuolo et al., 2007).

DHMEQ, a novel nuclear factor-kappaB inhibitor, induces selective depletion of alloreactive or phytohaemagglutinin-stimulated peripheral blood mononuclear cells, decreases production of T helper type 1 cytokines, and blocks maturation of dendritic cells (Nishioka et al., 2008).

Regarding the suppression of NF-kB by impaired IL-1 signaling, it was reported that delayed-type hypersensitivity (DTH) responses were significantly suppressed in IL-1b-deficient and IL-1a/b-deficient mice. Lymph node cells derived from antigen-sensitized IL-1b-deficient and IL-1a/b-deficient mice and IL-1R type I-deficient mice, exhibited reduced proliferative responses against antigen. These data suggest that IL-1b is necessary for the efficient priming of T cells. In addition, CD4+ T cell-derived IL-1 plays an important role in the activation of DCs during the elicitation phase, resulting in the production of TNF, that activate allergen-specific T cells (Nambu et al., 2006).

Quantitative Understanding of the Linkage

A representative NF-kB inhibitor, MG132 that suppresses NF-kB activity at more than 10 mM (Fiedler et al. 1998) suppresses IL-2-induced activation of STAT5 at 50 mM. (Yu and Malek 2001)
A representative NF-kB inhibitor, DHMEQ (1mg/mL) blocked PHA-induced nuclear translocation of NF-kB in Jurkat cells via inhibition of degradation of IkBa. Preincubation of peripheral blood mononuclear cells with DHMEQ (1 mg/ml, 3 hr) greatly reduced PHA-stimulated expression of IFN-g, IL-2 and TNF-a genes.

Response-response relationship
Interferon-γ (IFN-γ) production in response to CMV-infected fibroblasts was reduced under the influence of MG132 in a dose-dependent manner. A marked reduction was observed at 0.5 µM. Likewise, CMV-specific cytotoxicity of CD8(+) T cells was decreased in the presence of MG132 (Wang et al., 2011).

Bortezomib inhibits T-cell function versus infective antigenic stimuli in a dose-dependent manner in vitro (Orciuolo et al., 2007).

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Relationship: 2004: Suppression of T cell activation leads to Increase, Increased susceptibility to infection (https://aopwiki.org/relationships/2004)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of IL-1 binding to IL-1 receptor leading to increased susceptibility to infection (https://aopwiki.org/aops/277)	**adjacent**	**High**	**Not Specified**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

Normal T cell and B cell function is indispensable for host defense mechanism.

Evidence Supporting this KER

The experiments using knockout mice revealed that the lack of IL-1 signaling led to bacterial, tuberculosis or viral infection (Guler et al., 2011; Horino et al., 2009; Juffermans et al., 2000; Tian et al., 2017; Yamada et al., 2000).

Biological Plausibility
To protect the infection from different pathogens, different types of immune response depending on the pathogens are required.

1). Type 1 immunity drives resistance to viruses and intracellular bacteria, such as *Listeria monocytogenes*, *Salmonella* spp. and *Mycobacteria* spp., as well as to intracellular protozoan parasites such as *Leishmania* spp. The T helper 1 (T_H1) signature cytokine interferon-γ (IFNγ) has a central role in triggering cytotoxic mechanisms including macrophage polarization towards an antimicrobial response associated with the production of high levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS), activation of $CD8^+$ cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells to kill infected cells via the perforin and/or granzyme B-dependent lytic pathway or via the ligation of surface death receptors; and B cell activation towards the production of cytolytic antibodies that target infected cells for complement and Fc receptor-mediated cellular cytotoxicity.

2) Resistance to extracellular metazoan parasites and other large parasites is mediated and/or involves type 2 immunit. Pathogen neutralization is achieved via different mechanisms controlled by T_H2 signature cytokines, including interleukin-4 (IL-4), IL-5 and IL-13, and by additional type 2 cytokines such as thymic stromal lymphopoietin (TSLP), IL-25 or IL-33, secreted by damaged cell. T_H2 signature cytokines drive B cell activation towards the production of high-affinity pathogen-specific IgG1 and IgE antibodies that function via Fc-dependent mechanisms to trigger the activation of eosinophils, mast cells and basophils, expelling pathogens across epithelia.

3) T_H17 immunity confers resistance to extracellular bacteria such as *Klebsiella pneumoniae, Escherichia coli, Citrobacter rodentium, Bordetella pertussis*, *Porphyromonas gingivalis* and *Streptococcus pneumoniae,* and also to fungi such as *Candida albicans, Coccidioides posadasii, Histoplasma capsulatum* and *Blastomyces dermatitidis*. Activation of T_H17 cells by cognate T cell receptor (TCR–MHC class II interactions and activation of group 3 innate lymphoid cells (ILC3s) via engagement of IL-1 receptor (IL-1R) by IL-1β secreted from damaged cells lead to the recruitment and activation of neutrophils. T_H17 immunopathology is driven to a large extent by products of neutrophil activation, such as ROS and elastase (reviewed by Soares et al. (Soares et al., 2017)).

Based on these evidences, the insufficient T cell or B cell function causes impaired resistance to infection.

Empirical Evidence
Administration of IL-1R antagonist or neutralizing antibody such as IL-1Ra (generic anakinra), canakinumab (anti-IL-1β antibody) and rilonacept (soluble IL-1R) led to the suppression of downstream phenomena, which included internalization of IL-1 (Dripps et al., 1991), production of PGE_2 (Hannum et al., 1990; Seckinger et al., 1990), IL-6 (Goh et al., 2014), and T cell proliferation (Seckinger et al., 1990).

Since these inhibitors became available to treat some of autoinflammatory syndromes, it became clear that these inhibitors increased the frequency of serious bacterial infection (De Benedetti et al., 2018; Genovese et al., 2004; Imagawa et al., 2013; Kullenberg et al., 2016; Lachmann et al., 2009; Lequerre et al., 2008; Migkos et al., 2015; Schlesinger et al., 2012; Yokota et al., 2017).

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厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）

OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

令和元年度 分担研究報告書

Bhas42細胞形質転換試験法のTG開発

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研究要旨

Bhas42 細胞形質転換試験法（Bhas42CTA）は、化学物質の非遺伝毒性発がん性を遺伝毒性発がん性と区別して検出できる OECD 唯一の試験法（ガイダンスドキュメント掲載）である。OECD では、非遺伝毒性発がん性（NGTxC）検出を目的とした IATA（OECD NGTxC・IATA）開発が行われており、NGTxC の mode of action (MoA) が議論されている。2019 年度は、NGTxC・IATA としての国際合意に関するレビューの論文化に向けての意見集約および NGTxC・IATA の有害性発現経路（AOP: Adverse Outcome Pathway）の構成試験法のデータベースを構築し、Bhas42CTA の Test Guideline（TG）開発に繋げる活動を行った。

A. 研究目的

Bhas42細胞形質転換試験法（Bhas42CTA）は、化学物質の非遺伝毒性発がん性を遺伝毒性発がん性と区別して検出できるOECD唯一の試験法（ガイダンスドキュメント掲載）である。OECDでは、非遺伝毒性発がん性（NGTxC）検出を目的としたIATA（OECD NGTxC・IATA）開発が行われており、NGTxCのMoAが議論されている。そこで、NGTxC・IATAのexpert working groupとしてNGTxC・IATAの構築に貢献することにより、Bhas42CTAのTG開発に繋げる。

B. 研究方法

OECDのNGTxC・IATAのexpert working groupにより実施された4回の電話会議でNGTxC・IATAのレビューをまとめた。すなわち、発がんモデルをもとに複数のKey EventからなるMoAを構築した。各Key Eventに対応する試験法を選出し、詳細な情報をとりまとめたデータベースを作成し評価を行った。その際、Key Eventごとに13ブロックの評価チームを構成し、試験法の情報収集および評価をとりまとめた。大森は、Block 3の“Cell Transformation”とBlock 4の“Gap Junction”を担当した。

（倫理面への配慮）

当研究は、倫理審査およびCOIの指導・管理に該当しない。

C. 研究結果

OECDのNGTxC・IATAのexpert working groupで実施された電話会議では、2016年度から2018年度までの活動におけるNGTxC・IATAのレビューについて原案が提出され、それについて協議が行われた。その際、“Stepsconducive to the development of the

IATA for non-genotoxic carcinogen and assay evaluations”が全体方針として提案され、それに従いNGTxC・IATAの構築に向けた協議が行われた。

まず、発がんモデルとAOPをもとに複数のKey Eventからなる MoAを構築し、各Key Eventを定量的に評価可能な試験法を選出した。それらの詳細な情報をとりまとめ、データベースを作成し試験法のランキングを行った。その際、Key Eventごとに13ブロックの評価チームを組み、チームごとに情報収集および評価をとりまとめた。大森は、Block 3の“Cell Transformation”とBlock 4の“Gap Junction”を担当した。Block 4の“Gap Junction”チームでは、当初、Soft Agar AssayとVascular Permeability Assayが候補として他のメンバーから挙げられたが、“Gap Junction”はBlock 3の“Cell Transformation”、Vascular Permeability Assay はBlock 12の“Metastasis”への変更を大森から他のメンバーに提案し、“Gap Junction”としては、Dye Transfer AssayとInhibition of Metabolic Co-operation Assayを提案した。Dye Transfer Assayは、ECVAMでも推奨試験とされていたことから詳細な評価を行った。しかしながら、いずれのGap Junction Assayも研究室間再現性の評価の論文が存在せず、現時点ではOECDの試験法として組み入れるレベルには達していなかった。Block 3の“Cell Transformation”では、Bhas 42 CTAおよびSHE CTAはOECDでの評価済みガイダンスドキュメント掲載試験法として組み込まれた。

これら、OECDのNGTxC・IATAのexpert working groupがまとめたNGTxC・IATAのレビューは、Archives of Toxicologyに投稿し受理された。

D. 考察

今後もBhas42CTAの機序解析結果をNGTxC・IATAに提供することは、Bhas42CTAのTG開発に繋がる活動となるものと考える。

E. 結論

OECDのNGTxC・IATAのexpert working groupによりNGTxC・IATAが作成されており、Bhas 42 CTAは、OECDでの評価済みガイダンスドキュメント掲載試験法として、NGTxC・IATAを担う試験法として評価された。これらは、Bhas 42 CTAのTG開発に繋がり得る活動となった。

F. 研究発表

F.1. 論文発表

なし

F.2. 学会発表

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3. 福光徹, 大森清美; Bhas 42細胞形質転換試験法による農薬の発がんプロモーション活性の検討, 日本薬学会第140年会（紙上発表）

G. 知的財産権の出願・登録状況

G.1. 特許取得

なし

G.2. 実用新案登録

なし

G.3.その他

なし

厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）

OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

令和元年度 分担研究報告書

光毒性試験の AOP および IATA の開発

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研究要旨

外因性光線過敏症は近年注目を集める有害事情の一つであり，本毒性リスク回避のために効果的な予測方法の開発が国内外で急務の課題となっている。本研究では *in vitro* 光化学的試験方法である ROS アッセイを主軸とした AOP を作成するため，光毒性物質の光生物化学的ならびに光化学的特性を精査することで光毒性反応機序のさらなる解明を行った。また，得られた科学的根拠をベースに ROS assay は TG495 として OECD test guideline に採択され，OECD TG 化を達成した。

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A. 研究目的

近年、化合物の光安全性に対する関心の高まりから光毒性リスク評価に関する数多くの研究が行われている。ICH S10 で化合物の i) 光反応性および ii) 露光部位（皮膚や眼）への分布が光毒性発現に重要な因子として明記されている。当研究室では既に光化学的評価方法として reactive oxygen species (ROS) assay を開発し、本データと皮膚内動態情報の組み合わせることで信頼性ある光安全性評価が可能となることを明らかにした。この知見を検証すべく、本研究では ROS assay による光化学的特性および Franz 型拡散セルを用いた化学物質の *in vitro* 皮膚内動態のデータを統合的に解析することで経皮適用化合物の光毒性リスクを効果的に予測できるかを検証し、その予測データを用いることで動物実験代替法の開発を指向した検討を実施した。また、検証結果を基に光毒性に関する AOP ならびに ROS assay に関する OECD TG 案を作成した。なお、TG 案については協議の結果、OECD TG 495 としてガイドライン化に成功した。

B. 研究方法

B.1. ROS アッセイ

研究分担者らが既に公表している ROS assay 推奨プロトコルに基づき、quinolone derivatives (QNLs) 6 種 [enoxacin (ENX), flumequine (FLM), moxifloxacin (MFX), nalidixic acid (NLA), orbifloxacin (OFX), oxolinic acid (OXA)] について ROS assay を行った。

B.2. *In vitro* 皮膚内動態実験

上記 6 種の QNLs について、フランツ型拡散セルを用いてラット摘出皮膚における *in vitro* 皮膚透過性試験を実施した。ドナー側に QNLs (各 1 mg/mL) を入れ、経時的に皮膚を透過したレセプター液中の

QNLs の量を UPLC/ESI-MS にてモニタリングし、*in vitro* 皮膚透過性のデータを得た。得られたデータを基に定常状態における各 QNLs の皮膚内濃度 (C_{ss}) を算出した。得られた C_{ss} の値と光化学的特性データを併せて考慮することで光毒性予測を実施した。

B.3. ラット *in vivo* 光毒性試験

前日に腹部を剃毛した雄性ラットに各 QNLs (10 mg/site) を塗布し、塗布後 3 h で black light にて UVA (30 J/cm^2) を照射した。照射終了後 24 h に色差計にて皮膚表面の色調を計測し、光毒性の指標とした。

C. 研究結果

C.1. 光安全性評価

ROS assay にて 6 種の ONLs (ENX, FLM, MFX, NLA, OFX および OXA) は露光時に光安全性評価における ROS assay の criteria を超える強い ROS 産生を示し、高い光反応性を有していた。特に ENX は $^1O_2$ およびO_2^- ならびに OFX は $^1O_2$ において他の QNLs と比し強い ROS 産生を示した。*In vitro* 皮膚透過性を基に算出した C_{ss} は FLM および NLA がそれぞれ 5.0 および 8.2 μg/mL と高く、次いで MFX が 3.4 μg/mL であった。ENX, OFX および OXA の C_{ss} の値はそれぞれ 1.2, 2.0 および 2.2 μg/mL と比較的低値を示した。得られたデータを基に decision matrix を用いて統合的に 6 種の QNLs の光毒性リスク予測を実施した結果は以下の通りであった。

光毒性リスク予測：

NLA＞FLM＞OFX＞ENX＞OXA＞MFX

ラットにおける *in vivo* 皮膚光毒性について MFX を除く QNLs で UVA 非照射群と比し、UVA 照射群における有意な皮膚の色の変化を確認し、5 種の QNLs は光毒性陽性と判断した。MFX においては有意な皮膚の色の変化は認めず、*in vivo* 光毒性は弱いと判断した。QNLs の *in vivo* 光毒性の強さは以下の順であった。

In vivo 光毒性：

FLM＞NLA＞ENX≒OFX＞OXA＞MFX

C.2. AOP および OECD TG 案の作成

ROS assay の OECD TG 化のため、ROS assay の TG 案を提出し、各国から提示されたパブリックコメントに対応し、TG 改定案を作成した。その後、細かい箇所の修正を経て 2019 年 6 月に OECD TG495 としてガイドライン化に成功した。

D. 考察

本研究では光化学的特性および *in vitro* 皮膚内動態に基づき被験物質の光毒性リスクが予測可能か検証した。動物実験代替法として構築した ROS assay および *in vitro* 皮膚透過性を用いた光毒性予測系を構築し、6 種の QNLs の光毒性リスク予測を実施した結果、*in vivo* 光毒性の結果と良好に対応することが分かった。本研究で構築した評価手法は良好に光毒性リスク予測が可能であろう。

E. 結論

動物実験代替法としての光安全性評価系を構築し、光化学的特性ならびに *in vitro* 皮膚内濃度の統合的解析により良好に被験物質の光毒性リスクを予測できた。今回構築した光安全性評価系について更なる検証試験を進めるとともに、動物試料を用いない光安全性評価系構築を試みる。本検討で得られた知見は ROS assay の OECD TG 化の実現に大きく貢献した。さらに、現在提案中の光毒性に関する IATA 構築について

もこれまでに得られた知見は大きく貢献できると期待する。

F. 添付資料

AOP 282: Reactive oxygen species generated from photoreactive chemicals leading to phototoxic reactions

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H. 知的財産権の出願・登録状況

H.1. 特許取得

なし

H.2. 実用新案登録

なし

H.3. その他

なし

AOP ID and Title:

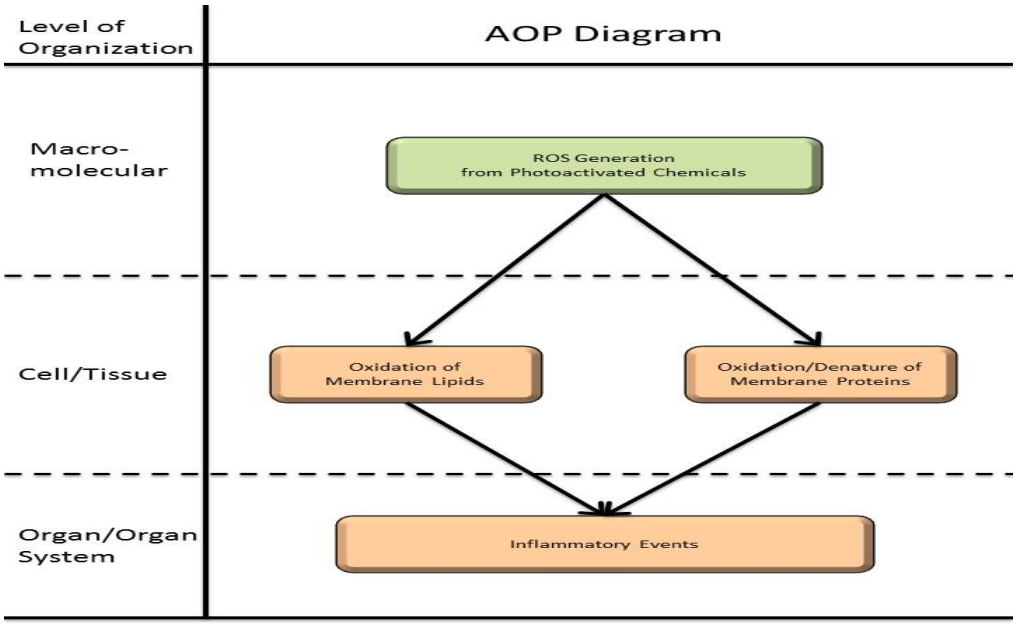
SNAPSHOT

Created at: 2020-05-26 00:39

AOP 282: Reactive oxygen species generated from photoreactive chemicals leading to phototoxic reactions

Short Title: ROS-mediated chemical phototoxicity

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	EAGMST Under Review	1.49	Included in OECD Work Plan

Abstract

Phototoxicity is an adverse reaction in the light-exposed tissues triggered by normally harmless doses of sunlight (Moore, 1998; Moore, 2002; Roberts, 2001). Recently, high-intensity UV rays from the sun have reached the Earth's surface with the destruction of the ozone layer, and interest in phototoxic events has increased enormously. Notably, phototoxic reactions against exogenous agents are caused by the combined effects of environmental light and external agents, including drugs, cosmetics, and foods (Epstein, 1983; Stein and Scheinfeld, 2007).

In this AOP, the primary trigger for a compound to be considered with respect to potential to create photochemical and photobiological reactions is the absorption of photon energy from light ranging from 290 to 700 nm. The extent of absorption depends on the wavelength of light and the type of absorbing chromophores in the light-exposed tissues. A molecule is excited by absorption of photon energy, and the photoactivated molecule

induces photochemical reactions via energy transfer (type I photochemical reaction) and free radical generation (type II photochemical reaction). These photochemical reactions result in generation of radicals and reactive oxygen species, and the reactive species react with biomolecules. Generated radicals of a target chemical bind to DNA and proteins, resulting in formation of these photo-adducts, and reactive oxygen species (ROS), including singlet oxygen and superoxide, induce oxidation of biomolecules. These key events bring inflammatory events in the light-exposed tissues (Brendler-Schwaab et al. , 2004; Epstein and Wintroub, 1985; Quintero and Miranda, 2000).

This AOP describes the pathway of photochemical toxicity between attack of ROS generated from photoactivated chemicals to membranes and inflammatory events in light-exposed tissues.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	1592	ROS generation from photoactivated chemicals (https://aopwiki.org/events/1592)	ROS generation
	KE	1594	Oxidation of membrane lipids (https://aopwiki.org/events/1594)	Oxidation of membrane lipids
	KE	1595	Oxidation/denatuation of membrane proteins (https://aopwiki.org/events/1595)	Oxidation/denatuation of membrane proteins
	AO	1599	Inflamatory events in light-exposed tissues (https://aopwiki.org/events/1599)	Inflammatory events

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
ROS generation from photoactivated chemicals (https://aopwiki.org/relationships/1845)	adjacent	Oxidation of membrane lipids	High	Low
ROS generation from photoactivated chemicals (https://aopwiki.org/relationships/1846)	adjacent	Oxidation/denatuation of membrane proteins	High	Low
Oxidation of membrane lipids (https://aopwiki.org/relationships/1850)	adjacent	Inflamatory events in light-exposed tissues	High	Low
Oxidation/denatuation of membrane proteins (https://aopwiki.org/relationships/1851)	adjacent	Inflamatory events in light-exposed tissues	High	Low

Stressors

Name	Evidence
Light (290-700 nm)	High
Photoreactive chemicals	High
Reactive oxygen species	High

Overall Assessment of the AOP

The focus of this AOP is on photochemical toxicity, especially photoactivation of target chemicals followed by generation of ROS. ROS generated from photoirradiated chemicals can react with molecules on the membranes, including lipids and proteins, and the reactions may lead to inflammatory events in the UV-exposed tissues.

Phototoxicity is an adverse reaction triggered by normally harmless doses of sunlight. There are two types of photosensitive disorders, endogenous and exogenous phototoxicity, and the observable changes to the sunlight-exposed tissues are essentially detrimental, and include the following appearance; (i) immediate faint erythema during exposure, (ii) delayed erythemal responses, (iii) abnormal keratinisation and vacuolated cells, (iv) formation of desquamating layer, and (v) desquamation (peeling) (Moore, 1998; Moore, 2002; Roberts, 2001). Recently, high-intensity UV rays from the sun have reached the Earth's surface with the destruction of the ozone layer, and interest in phototoxic events has increased enormously. Notably, phototoxic reactions against exogenous agents are caused by the combined effects of UV irradiation and external agents, including drugs, cosmetics and foods (Stein and Scheinfeld, 2007). Phototoxic skin responses after administration of photosensitive drugs, so-called drug-induced phototoxicity, have been recognized as undesirable side effects, and several classes of drugs, even when not toxic by themselves, may become reactive under exposure to environmental light, inducing undesired phototoxic responses (Epstein, 1983).

The primary trigger for a compound to be considered with respect to potential to create photochemical and photobiological reaction is the absorption of UV and visible light ranging from 290 to 700 nm. The extent of absorption depends on the wavelength of light and the type of absorbing chromophores in the UV-exposed tissues. UV radiation is usually divided into several ranges based on its physiologic effects: (1) UVA (near UV): 320–400 nm (UVA I: 340–400 nm and UVA II: 320–340 nm), (2) UVB (middle UV): 290–320 nm, and (3) UVC (far UV): 180–290 nm (Svensson et al., 2001; Vassileva et al., 1998). The sun emits ultraviolet radiation in the UVA, UVB, and UVC bands, but because of absorption by the atmosphere's ozone layer, the main ultraviolet radiation that reaches the Earth's surface is UVA (Dubakiene and Kupriene, 2006). Absorption of light through the skin and eyes, primarily in the 290–700 nm range, varies with wavelength, such that light in the red region of the spectrum reaches well into the subcutis layer; whereas at 300 nm or shorter wavelength, only an estimated 10% passes through the epidermis (Epstein, 1989). Thus, penetration and absorption of light in the UV-exposed tissues is important factor in drug-induced phototoxicity as Grotthus-Draper law of photobiology states; only light that is absorbed can be active in photochemical and photobiological processes.

When a drug molecule absorbs a photon energy, electrons can be prompted from occupied orbitals (the ground state) to an unoccupied orbital (S1, S2) depending upon bond type and associated energy level. Furthermore, unpaired singlet state electrons (opposite spin) may be converted to triplet state (parallel spin) by inversion of the spin via intersystem crossing of the absorbed energy. To return to the ground state from S1, S2/T1, T2, energy must be dissipated by internal conversion, fluorescence (from singlet state), phosphorescence (from triplet state) or via chemical reaction, giving rise to photoproducts and/or potential external reactions with biomolecules.

In addition, molecular oxygen, a triplet radical in its ground state, appears to be the predominant acceptor of excitation energy as its lowest excited level (singlet state) has a comparatively low value. An energy transfer from excited triplet photosensitizer to the oxygen (type II photochemical reaction) could produce excited singlet oxygen which might, in turn, participate in a lipid- and protein-membrane oxidation or induce DNA damage. An electron or hydrogen transfer could lead to the formation of free radical species (type I photochemical reaction), producing a direct attack on the biomolecules or in the presence of oxygen, to evolve towards secondary free radicals such as peroxyl radicals or the very reactive hydroxyl radical, a known intermediate in the oxidative damage of biomolecules. This toxic pathway corresponds to successive reactions which involve the appearance of superoxide anion radical, its dismutation to from hydrogen peroxide followed with the hydrogen peroxide reduction to form hydroxyl radical. Herein, excitation of the drug by light may give rise to ROS such as singlet oxygen and superoxide, which may be one of causative molecules for the drug-induced phototoxicity (Brendler-Schwaab et al., 2004; Epstein and Wintroub, 1985).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Sex Applicability

Sex	Evidence
Mixed	High

Chemicals: This AOP applies to a wide range of chemicals. Phototoxic chemicals are recognized to have following characteristics: (i) absorption of light within the range of natural sunlight (290-700 nm); (ii) generation of a reactive species following absorption of UV-visible light; (iii) distribution to light-exposed tissues (e.g., skin and eye) (ICH S10).

Sex: This AOP applies to both males and females.

Life stages: The relevant life stages for this AOP are all life stages after born.

Taxonomic: This AOP mainly applies to human.

Essentiality of the Key Events

The essentiality of KEs for this AOP was rated high on the basis of experimental evidence in the investigations related to each of KEs and published guidelines. For details see the table on “Support for Essentiality of KEs”.

Weight of Evidence Summary

Support for biological plausibility of KERs

MIE => KE 1	Generated ROS from photoactivated chemicals can react with membrane lipids, and oxidation of membrane lipids could be occurred.	Biological Plausibility of the MIE => KE 1 is high. The relationship between MIE and KE 1 is consistent with chemical and biological knowledge (Girotti, 1990; Girotti, 2001; Onoue and Tsuda, 2006).
MIE => KE 2	Generated ROS from photoactivated chemicals can react with membrane proteins, and oxidation/denaturation of membrane proteins could be occurred.	Biological Plausibility of the MIE => KE 2 is high. The relationship between MIE and KE 2 is consistent with biological knowledge (Dalle Carbonare and Pathak, 1992; Valenzeno, 1987).
KE 1 => AO	Oxidation of membrane lipids relates with damage produced in the cellular membrane, leading to inflammatory events.	Biological Plausibility of the KE 1 => AO is high. The relationship between KE 1 and AO is consistent with biological knowledge (Castell et al., 1994).
KE 2 => AO	Oxidation/denaturation of protein provides the necrosis of the living tissues as an inflammatory event.	Biological Plausibility of the KE 2 => AO is high. The relationship between KE 2 and AO is consistent with biological knowledge (Dalle Carbonare and Pathak, 1992; Opie, 1962).

Support for Essentiality of KEs

MIE	ROS generation from photoactivated chemicals	High; well-accepted generation of reactive oxygen species from photo-activated chemicals associated with phototoxic reactions with 200 of chemicals evaluated in qualitative endpoints (Onoue et al., 2014; Onoue et al., 2013a; Onoue et al., 2008a; Onoue and Tsuda, 2006; Seto et al., 2013b). The event has described in ICH S10 guideline as a crucial factor of phototoxic reactions (ICH, 2014).
KE 1	Oxidation of membrane lipids	High; Oxidative stress to lipids associated with the phototoxic reactions (Girotti, 1990; Girotti, 2001; Onoue and Tsuda, 2006).
KE 2	Oxidation/denaturation of membrane proteins	High; accepted oxidation/denaturation of proteins associated with the phototoxic reactions (Dalle Carbonare and Pathak, 1992; Valenzeno, 1987).
Adverse outcome	Inflammatory events in sunlight-exposed tissues	Photoreactive agents indicated inflammatory events, including edema, dyskeratosis, and necrosis, in light-exposed tissues after sunlight exposure (Moore, 1998; Moore 2002; Roberts, 2001).

Empirical Support for KERs

MIE => KE 1: ROS generation leads to Oxidation of membrane lipids	Empirical support of the MIE => KE 1 is strong. Rationale: Lipid peroxidation was occurred by ROS-generating chemicals under exposure to simulated sunlight (Onoue et al., 2011, Onoue and Tsuda, 2006). A photoreactive chemical indicated dose-dependent increases in ROS generation and lipid peroxidation after exposure to a fixed dose of simulated sunlight (Seto et al., 2013a).
MIE => KE 2: ROS generation leads to Oxidation/denaturation of membrane proteins	Empirical support of the MIE => KE 2 is moderate. Rationale: ROS generated from photosensitizing agents led to oxidation and denaturation of proteins (Dalle Carbonare and Pathak, 1992).
KE 1 => AO: Oxidation of membrane lipids leads to Inflammatory events	Empirical support of the KE 1=> AO is strong. Rationale: Increases in lipid peroxidation and inflammatory-related cytokines were observed in the murine skin, and naringenin, an anti-oxidant, attenuated these increases in a dose-dependent manner (Martinez et al., 2015). Benzoyl peroxide, a ROS generator, led to lipid peroxidation and GSH depletion, and the changes caused the gene expression of pro-inflammatory cytokines (Valacchi et al., 2001).

KE 2 => AO: Oxidation/denaturation of membrane proteins leads to Inflammatory events	Empirical support of the KE 2=> AO is moderate. Rationale: Denaturation of proteins induced necrosis and inflammatory in the skin (Opie, 1962).

Quantitative Consideration

Although there is empirical information on KERs as described above sections, the overall quantitative understanding of the AOP is insufficient to directly link a measure of KEs to a quantitative prediction of KERs.

As a pre-MIE, light absorption of chemicals is an important event for phototoxic reactions induced by photoreactive chemicals. Quantitative endpoint on absorption of light (290–700 nm) was recognized in the previous report (Henry et al., 2009), and, for photoreactive chemicals, the criterion on molar extinction coefficient (MEC) was determined to be 1,000 $M^{-1}\cdot cm^{-1}$. Most of chemicals with MEC values of over 1,000 $M^{-1}\cdot cm^{-1}$ generated significant ROS, including singlet oxygen and/or superoxide (Onoue et al., 2013b; Onoue and Tsuda, 2006), and the qualitative criteria on ROS generation was determined to evaluate chemical phototoxicity (Onoue et al., 2014; Onoue et al., 2013a; Onoue et al., 2008b).

Considerations for Potential Applications of the AOP (optional)

The MIE and KEs in this AOP could contribute to assays development for photosafety evaluation and an AOP-based IATA construction. AOP-based IATA can be applied for various aims including screening of chemicals, prioritization of chemicals for further testing, and risk assessment.

The regulatory applicability of the AOP would be to use experimental results from assays based on MIE and KEs as indictors for the risk of phototoxic reactions.

Combined use of photobiochemical properties and tissue exposure data would be of help for photosafety evaluation of chemicals. Risk assessment would be possible when exposure data in light-exposed tissues combined with assay data based on AOP.

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Appendix 1

List of MIEs in this AOP

Event: 1592: ROS generation from photoactivated chemicals (https://aopwiki.org/events/1592)

Short Name: ROS generation

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:282 - Reactive oxygen species generated from photoreactive chemicals leading to phototoxic reactions (https://aopwiki.org/aops/282)	MolecularInitiatingEvent

Stressors

Name
Light (290-700 nm)
Photoreactive chemicals

Biological Context

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Several classes of chemicals cause ROS generation under light exposure, and the ROS generation can be monitored by ROS assay (Onoue et al., 2014, Onoue et al., 2013, Onoue et al. , 2008, Seto et al. , 2013). The criteria of ROS assay for photosafety assessment of chemicals were defined (Onoue et al., 2014, Onoue et al., 2013).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Chemicals: This MIE applies to a wide range of chemicals. The chemicals absorb photon energy from light within the range of natural sunlight (290-700 nm) (ICH, 2014, Onoue and Tsuda, 2006).

Sex: This MIE applies to both males and females.

Life stages: The relevant life stages for this MIE are all life stages after born.

Taxonomic: This MIE mainly applies to human.

Key Event Description

In the primary event, photoreactive chemicals are excited by the absorption of photon energy. The energy of the photoactivated chemicals transfer to oxygen and then generates the reactive oxygen species (ROS), including superoxide (O_2^-) via type I reaction and singlet oxygen ($^1O_2$) via type II reaction, as principal intermediate species in phototoxic reaction (Foote, 1991, Onoue et al. , 2009).

How it is Measured or Detected

On the basis of the pathogenesis of drug-induced phototoxicity, a reactive oxygen species (ROS) assay was proposed to evaluate the phototoxic risk of chemicals. The ROS assay can monitor generation of ROS, such as singlet oxygen and superoxide, from photoirradiated chemicals, and the ROS data can be used to evaluate the photoreactivity of chemicals (Onoue et al. , 2014, Onoue et al. , 2013, Onoue and Tsuda, 2006). The ROS assay is a recommended approach by guidelines to evaluate the phototoxic risk of chemicals (ICH, 2014, PCPC, 2014).

References

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ICH. ICH Guideline S10 Guidance on Photosafety Evaluation of Pharmaceuticals.: International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2014.

Onoue S, Hosoi K, Toda T, Takagi H, Osaki N, Matsumoto Y, et al. Intra-/inter-laboratory validation study on reactive oxygen species assay for chemical photosafety evaluation using two different solar simulators. Toxicology in vitro : an international journal published in association with BIBRA. 2014;28:515-23.

Onoue S, Hosoi K, Wakuri S, Iwase Y, Yamamoto T, Matsuoka N, et al. Establishment and intra-/inter-laboratory validation of a standard protocol of reactive oxygen species assay for chemical photosafety evaluation. Journal of applied toxicology : JAT. 2013;33:1241-50.

Onoue S, Kawamura K, Igarashi N, Zhou Y, Fujikawa M, Yamada H, et al. Reactive oxygen species assay-based risk assessment of drug-induced phototoxicity: classification criteria and application to drug candidates. J Pharm Biomed Anal. 2008;47:967-72.

Onoue S, Seto Y, Gandy G, Yamada S. Drug-induced phototoxicity; an early *in vitro* identification of phototoxic potential of new drug entities in drug discovery and development. Current drug safety. 2009;4:123-36.

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Seto Y, Kato M, Yamada S, Onoue S. Development of micellar reactive oxygen species assay for photosafety evaluation of poorly water-soluble chemicals. Toxicology in vitro : an international journal published in association with BIBRA. 2013;27:1838-46.

List of Key Events in the AOP

Event: 1594: Oxidation of membrane lipids (https://aopwiki.org/events/1594)

Short Name: Oxidation of membrane lipids

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:282 - Reactive oxygen species generated from photoreactive chemicals leading to phototoxic reactions (https://aopwiki.org/aops/282)	KeyEvent

Stressors

Name
Photoactivated chemicals
Reactive oxygen species

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Chemicals: This KE applies to a wide range of chemicals. The chemicals generate a reactive species, such as reactive oxygen species, following absorption of photon energy from light within the range of natural sunlight (290-700 nm) (ICH, 2014, Onoue and Tsuda, 2006).

Sex: This KE applies to both males and females.

Life stages: The relevant life stages for this KE are all life stages after born.

Taxonomic: This KE mainly applies to human.

Key Event Description

Lipid peroxidation of membrane lipids has been considered to be one of the major mechanisms in phototoxic skin responses induced by photoreactive chemicals (Castell et al., 1994, Girotti, 1990, 2001, Onoue et al., 2011, Onoue and Tsuda, 2006, Seto et al., 2013).

How it is Measured or Detected

An *in vitro* system using thiobarbituric acid can monitor lipid peroxidation by photoactivated chemicals (Onoue et al., 2011, Onoue and Tsuda, 2006).

References

Castell JV, Gomez-Lechon MJ, Grassa C, Martinez LA, Miranda MA, Tarrega P. Photodynamic lipid peroxidation by the photosensitizing nonsteroidal antiinflammatory drugs suprofen and tiaprofenic acid. Photochem Photobiol. 1994;59:35-9.

Girotti AW. Photodynamic lipid peroxidation in biological systems. Photochem Photobiol. 1990;51:497-509.

Girotti AW. Photosensitized oxidation of membrane lipids: reaction pathways, cytotoxic effects, and cytoprotective mechanisms. J Photochem Photobiol B. 2001;63:103-13.

ICH. ICH Guideline S10 Guidance on Photosafety Evaluation of Pharmaceuticals.: International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2014.

Onoue S, Seto Y, Ochi M, Inoue R, Ito H, Hatano T, et al. In vitro photochemical and phototoxicological characterization of major constituents in St. John's Wort (Hypericum perforatum) extracts. Phytochemistry. 2011;72:1814-20.

Onoue S, Tsuda Y. Analytical studies on the prediction of photosensitive/phototoxic potential of pharmaceutical substances. Pharmaceutical research. 2006;23:156-64.

Seto Y, Inoue R, Kato M, Yamada S, Onoue S. Photosafety assessments on pirfenidone: photochemical, photobiological, and pharmacokinetic characterization. J Photochem Photobiol B. 2013;120:44-51.

Event: 1595: Oxidation/denatuation of membrane proteins (https://aopwiki.org/events/1595)

Short Name: Oxidation/denatuation of membrane proteins

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:282 - Reactive oxygen species generated from photoreactive chemicals leading to phototoxic reactions (https://aopwiki.org/aops/282)	KeyEvent

Stressors

Name
Photoactivated chemicals
Reactive oxygen species

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Chemicals: This KE applies to a wide range of chemicals. The chemicals generate a reactive species, such as reactive oxygen species, following absorption of photon energy from light within the range of natural sunlight (290-700 nm) (ICH, 2014, Onoue and Tsuda, 2006).

Sex: This KE applies to both males and females.

Life stages: The relevant life stages for this KE are all life stages after born.

Taxonomic: This KE mainly applies to human.

Key Event Description

Reactive oxygen species yielded from photoactivated chemicals can cause cross-linking of proteins and oxidation of sulfydryl groups resulting in disulfide cross-links (Dalle Carbonare and Pathak, 1992).

How it is Measured or Detected

As for in vitro systems, sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and other gel electrophoresis methodologies can detect denaturation of proteins (Dalle Carbonare and Pathak, 1992).

References

Dalle Carbonare M, Pathak MA. Skin photosensitizing agents and the role of reactive oxygen species in photoaging. J Photochem Photobiol B. 1992;14:105-24.

ICH. ICH Guideline S10 Guidance on Photosafety Evaluation of Pharmaceuticals.: International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2014.

Onoue S, Tsuda Y. Analytical studies on the prediction of photosensitive/phototoxic potential of pharmaceutical substances. Pharmaceutical research. 2006;23:156-64.

List of Adverse Outcomes in this AOP

Event: 1599: Inflamatory events in light-exposed tissues (https://aopwiki.org/events/1599)

Short Name: Inflammatory events

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:282 - Reactive oxygen species generated from photoreactive chemicals leading to phototoxic reactions (https://aopwiki.org/aops/282)	AdverseOutcome

Stressors

Name
Light (290-700 nm)
Photoreactive chemicals
Reactive oxygen species

Biological Context

Level of Biological Organization
Organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Chemicals: This AO applies to a wide range of chemicals. Phototoxic chemicals are recognized to have following characteristics: (i) absorption of light within the range of natural sunlight (290-700 nm); (ii) generation of a reactive species following absorption of UV-visible light; (iii) distribution to light-exposed tissues (e.g., skin and eye) in ICH S10 guideline for photosafety assessment (ICH, 2014).

Sex: This AO applies to both males and females.

Life stages: The relevant life stages for this AO are all life stages after born.

Taxonomic: This AO mainly applies to human.

Key Event Description

Photoirritation is frequently characterized as exaggerated sunburn sometimes mediated by oxidative stress in the cell membrane, and hyperpigmentation and desquamation may occur as a residual effect of a phototoxic reaction. Theoretically, if a high concentration of a phototoxic drug accumulates in the skin and the appropriate wavelength of light is present, any individual will develop a phototoxic reaction. In particular, peroxidation of membrane lipid could be induced by some photosensitizers under photo-irradiation, and this photochemical reaction certainly correlates with damage produced in the cell membrane, leading to the skin photoirritation (Castell et al. , 1994, Onoue et al. , 2009).

How it is Measured or Detected

Inflammatory events induced by photoreactive chemicals can be detected *in vivo* phototoxicity testing and photopatch test in clinical (Epstein, 1964, ICH, 2014, Onoue et al., 2009).

Regulatory Significance of the AO

Inflammatory events in light-exposed tissues are considered to be the endpoint of ROS-mediated chemical phototoxicity, especially photoirritant reactions (ICH, 2014, Onoue et al., 2009).

References

Castell JV, Gomez-Lechon MJ, Grassa C, Martinez LA, Miranda MA, Tarrega P. Photodynamic lipid peroxidation by the photosensitizing nonsteroidal antiinflammatory drugs suprofen and tiaprofenic acid. Photochem Photobiol. 1994;59:35-9.

Epstein S. The Photopatch Test; Its Technique, Manifestations, and Significance. Ann Allergy. 1964;22:1-11.

ICH. ICH Guideline S10 Guidance on Photosafety Evaluation of Pharmaceuticals.: International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2014.

Onoue S, Seto Y, Gandy G, Yamada S. Drug-induced phototoxicity; an early *in vitro* identification of phototoxic potential of new drug entities in drug discovery and development. Current drug safety. 2009;4:123-36.

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1845: ROS generation leads to Oxidation of membrane lipids (https://aopwiki.org/relationships/1845)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Reactive oxygen species generated from photoreactive chemicals leading to phototoxic reactions (https://aopwiki.org/aops/282)	**adjacent**	**High**	**Low**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Chemicals: This KER applies to a wide range of chemicals. The chemicals absorb photon energy from light within the range of light (290-700 nm) (ICH, 2014, Onoue and Tsuda, 2006).

Sex: This KER applies to both males and females.

Life stages: The relevant life stages for this KER are all life stages after born.

Taxonomic: This KER mainly applies to human.

Key Event Relationship Description

Some photoactivated chemicals can generate reactive oxygen species (ROS) after photoactivation of chemicals by irradiation of light (290–700 nm). ROS generated from photoactivated chemicals can react with membrane lipids and lead to oxidation of membrane lipids.

Evidence Supporting this KER

Biological Plausibility

Photoactivated chemicals generate ROS, and the ROS-generating chemicals cause lipid peroxidation under exposure to light (290–700 nm) in chemical and biological systems (Girotti, 1990, 2001, Onoue and Tsuda, 2006).

Empirical Evidence

Lipid peroxidation was occurred by ROS-generating chemicals under exposure to simulated sunlight (Onoue et al. , 2011, Onoue and Tsuda, 2006).

A photoreactive chemical indicated dose-dependent increases in ROS generation and lipid peroxidation after exposure to a fixed dose of simulated sunlight (Seto et al. , 2013).

References

Girotti AW. Photodynamic lipid peroxidation in biological systems. Photochem Photobiol. 1990;51:497-509.

Girotti AW. Photosensitized oxidation of membrane lipids: reaction pathways, cytotoxic effects, and cytoprotective mechanisms. J Photochem Photobiol B. 2001;63:103-13.

ICH. ICH Guideline S10 Guidance on Photosafety Evaluation of Pharmaceuticals.: International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2014.

Onoue S, Seto Y, Ochi M, Inoue R, Ito H, Hatano T, et al. In vitro photochemical and phototoxicological characterization of major constituents in St. John's Wort (Hypericum perforatum) extracts. Phytochemistry. 2011;72:1814-20.

Onoue S, Tsuda Y. Analytical studies on the prediction of photosensitive/phototoxic potential of pharmaceutical substances. Pharmaceutical research. 2006;23:156-64.

Seto Y, Inoue R, Kato M, Yamada S, Onoue S. Photosafety assessments on pirfenidone: photochemical, photobiological, and pharmacokinetic characterization. J Photochem Photobiol B. 2013;120:44-51.

Relationship: 1846: ROS generation leads to Oxidation/denatuation of membrane proteins (https://aopwiki.org/relationships/1846)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Reactive oxygen species generated from photoreactive chemicals leading to phototoxic reactions (https://aopwiki.org/aops/282)	**adjacent**	**High**	**Low**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Chemicals: This KER applies to a wide range of chemicals. The chemicals absorb photon energy from light within the range of light (290-700 nm) (ICH, 2014, Onoue and Tsuda, 2006).

Sex: This KER applies to both males and females.

Life stages: The relevant life stages for this KER are all life stages after born.

Taxonomic: This KER mainly applies to human.

Key Event Relationship Description

Some photoactivated chemicals can generate reactive oxygen species (ROS) after photoactivation of chemicals by irradiation of light (290–700 nm). ROS generated from photoactivated chemicals can react with membrane proteins and lead to oxidation/denaturation of membrane proteins.

Evidence Supporting this KER

Biological Plausibility
Photoactivated chemicals by UVA generate ROS including singlet oxygen, superoxide, and hydroxyl radicals, and the generated ROS cause cross-linking of proteins and denaturation of proteins as oxidative damages by photoactivated chemicals (Dalle Carbonare and Pathak, 1992).

Empirical Evidence
ROS generated from photosensitizing agents led to oxidation and denaturation of proteins, resulting in cross-linking of proteins and oxidation of sulfydryl groups (Dalle Carbonare and Pathak, 1992).

References

Dalle Carbonare M, Pathak MA. Skin photosensitizing agents and the role of reactive oxygen species in photoaging. J Photochem Photobiol B. 1992;14:105-24.

ICH. ICH Guideline S10 Guidance on Photosafety Evaluation of Pharmaceuticals.: International Council on Harmonisation of Technical

Requirements for Registration of Pharmaceuticals for Human Use; 2014.

Onoue S, Tsuda Y. Analytical studies on the prediction of photosensitive/phototoxic potential of pharmaceutical substances. Pharmaceutical research. 2006;23:156-64.

Relationship: 1850: Oxidation of membrane lipids leads to Inflammatory events (https://aopwiki.org/relationships/1850)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Reactive oxygen species generated from photoreactive chemicals leading to phototoxic reactions (https://aopwiki.org/aops/282)	**adjacent**	**High**	**Low**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Chemicals: This KER applies to a wide range of chemicals. The chemicals generate ROS under exposure to light (290-700 nm) (ICH, 2014, Onoue and Tsuda, 2006).

Sex: This KER applies to both males and females.

Life stages: The relevant life stages for this KER are all life stages after born.

Taxonomic: This KER mainly applies to human.

Key Event Relationship Description

Oxidation of membrane lipids can lead to inflammatory events via oxidative damage of cellular membranes and increases in expression of pro-inflammatory cytokines.

Evidence Supporting this KER

Biological Plausibility

Lipid peroxidation in cellar membrane is an oxidative damage of cellular membrane, and viability of cells decreases by lipid peroxidation of photoreactive chemicals (Castell et al., 1994).

Empirical Evidence

Increases in lipid peroxidation and inflammatory-related cytokines were observed in the murine skin, and naringenin, an anti-oxidant, attenuated these increases in a dose-dependent manner (Martinez et al., 2015).

Benzoyl peroxide, a ROS generator, led to lipid peroxidation and GSH depletion, and the changes caused the gene expression of pro-inflammatory cytokines in human keratinocytes (Valacchi et al., 2001).

References

Castell JV, Gomez-Lechon MJ, Grassa C, Martinez LA, Miranda MA, Tarrega P. Photodynamic lipid peroxidation by the photosensitizing nonsteroidal antiinflammatory drugs suprofen and tiaprofenic acid. Photochem Photobiol. 1994;59:35-9.

ICH. ICH Guideline S10 Guidance on Photosafety Evaluation of Pharmaceuticals.: International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2014.

Martinez RM, Pinho-Ribeiro FA, Steffen VS, Caviglione CV, Vignoli JA, Barbosa DS, et al. Naringenin Inhibits UVB Irradiation-Induced Inflammation and Oxidative Stress in the Skin of Hairless Mice. Journal of natural products. 2015;78:1647-55.

Onoue S, Tsuda Y. Analytical studies on the prediction of photosensitive/phototoxic potential of pharmaceutical substances. Pharmaceutical research. 2006;23:156-64.

Valacchi G, Rimbach G, Saliou C, Weber SU, Packer L. Effect of benzoyl peroxide on antioxidant status, NF-kappaB activity and interleukin-1alpha gene expression in human keratinocytes. Toxicology. 2001;165:225-34.

Relationship: 1851: Oxidation/denatuation of membrane proteins leads to Inflammatory events (https://aopwiki.org/relationships/1851)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Reactive oxygen species generated from photoreactive chemicals leading to phototoxic reactions (https://aopwiki.org/aops/282)	**adjacent**	**High**	**Low**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Chemicals: This KER applies to a wide range of chemicals. The chemicals generate ROS under exposure to light (290-700 nm) (ICH, 2014, Onoue and Tsuda, 2006).

Sex: This KER applies to both males and females.

Life stages: The relevant life stages for this KER are all life stages after born.

Taxonomic: This KER mainly applies to human.

Key Event Relationship Description

Oxidation/denaturation of membrane proteins can lead to inflammatory events via oxidative damage of cellular membranes and increases in expression of pro-inflammatory cytokines.

Necrosis in living tissues can be occurred as an inflammatory events via oxidative damages of membrane proteins.

Evidence Supporting this KER

Biological Plausibility
Oxidative damage of membrane proteins by photoactivated chemicals can cause oxidation and denaturation of proteins, and damaged proteins induces inflammatory events (necrosis) (Dalle Carbonare and Pathak, 1992, Opie, 1962).

Empirical Evidence
Denaturation of proteins induced necrosis and inflammatory in the skin (Opie, 1962).

References

Dalle Carbonare M, Pathak MA. Skin photosensitizing agents and the role of reactive oxygen species in photoaging. J Photochem Photobiol B. 1992;14:105-24.

ICH. ICH Guideline S10 Guidance on Photosafety Evaluation of Pharmaceuticals.: International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2014.

Onoue S, Tsuda Y. Analytical studies on the prediction of photosensitive/phototoxic potential of pharmaceutical substances. Pharmaceutical research. 2006;23:156-64.

Opie EL. On the relation of necrosis and inflammation to denaturation of proteins. The Journal of experimental medicine. 1962;115:597-608.

厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）

OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

令和元年度 分担研究報告書

免疫毒性のAOP開発

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研究要旨

日本免疫毒性学会 AOP 検討小委員会とともに、免疫毒性に関する 4 種の AOP の開発を行った。

すでに AOP wiki に登録済みである「Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response」については、今年度外部レビューに進み、外部レビュアーの指摘に基づいて AOP 案の修正を行った。

昨年度 OECD に SPSF（Standard Project Submission Form）を提出し、計画が承認された以下 3 種の AOP はコーチ制となり、それぞれのコーチとともに AOP の作成を行った。

「Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease」については、提出した AOP 案に対するコーチのコメントに基づいて修正案を作成中である。

「Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus」については、コーチと 2 回の web 会議を行い、AOP 案の修正を行っている。

「Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response」については、コーチと 3 回の会議を行い、AOP 案の修正を行っている。

A. 研究目的

免疫毒性は化学物質の安全性を評価するうえで重要な項目であり、その複雑さからIATA (Integrated Approaches to Testing and Assessment)による総合的な評価が必要と考えられている。IATA確立のためには、免疫毒性に関する複数のAOPを開発し、ネットワーク化する必要がある。日本主導でAOPを開発することにより、世界中の人々の化学物質による免疫毒性被害の防止に貢献するだけでなく、日本の研究レベルの高さを世界にアピールすることを目的とする。

B. 研究方法

AOP案の作成は、日本免疫毒性学会会員をメンバーとする同学会試験法委員会AOP検討小委員会に委託した（研究分担者も本小委員会の委員として活動）。それぞれ開発進度に合わせて、OECDより任命された外部レビュアーあるいはコーチと意見交換を行い、最終化に向けて修正を行う。

（倫理面への配慮）

本研究は動物実験を含め新たな実験は行わないため、倫理的問題は無いと考える。

C. 研究結果

「Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response」は、カルシニューリン阻害によりIL-2および４の産生抑制が生じ、最終的にTDAR (T-cell dependent antibody response)の阻害となるとするAOPである。本AOPについては、内部レビュアーによるマイナーコメントへの対応を行った結果、2019年6月にOECDパリ本部にて開催されたEAGMST (Advisory Group on Molecular Screening and Toxicogenomics)会議において、外部レビュー移行となった。その後外部レビュアーから詳細なコメントがあり、それらに基づいてAOPの修正を行った。主な論点とその対応を以下に示す。本AOPのMIE (Molecular Initiating Point)はカルシニューリンの阻害であり、免疫抑制剤であるFK506やサイクロスポリンをストレッサーとして挙げていたが、一般化学物質も例示すべきとの意見に従い、IC50は高くなるもののケンペロールなどカルシニューリン阻害活性がある化学物質を追加した。また、IL-2とIL-4の産生抑制がAO (Adverse Outcome)であるTDRAにつながるとした点について、IL-4の抑制がIgEクラスのみの産生を抑制するデータであったことから、関係性が弱いとの指摘があった。そこでIL-4を削除してIL-2のみの記載にすることも検討したが、その後の文献調査により、IL-4の抑制によるIgMおよびIgGクラスの抑制も明らかとなったことから、IL-4の産生抑制もTDARの抑制要因として残すこととした。

「Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease」は、樹状細胞に存在するTLR (Toll-like receptor) 7/8への結合が、Th17によるIL-17の過剰発現を誘導し、乾癬様の皮膚疾患を生じさせるというAOPである。本AOPについては、提出したAOP案に対するコーチのコメントへの対応案を作成した。主な論点とその対応を以下に示す。当初、TLR (Toll Like Receptor)7/8への刺激が、樹状細胞のIL-23の過剰産生につながるとしていたが、より一般的な炎症のKE (Key Event)をその間に入れることで規制での活用においてより重要性が高くなるという指摘があり、樹状細胞の活性化をKEとして追加することを検討している。また、例えば環境汚染物質などの潜在的なストレッサーを考慮するべきとの指摘については、TLR 7/8のリガンドとして知られている一本鎖RNAやグアノシンなどの低分子化合物から構造的特徴が見いだせないか検討中である。

「Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus」は、様々なタイプの免疫細胞に存在するERの活性化がTh2タイプのサイトカイン(IL-4)の異常亢進を誘導し、自己免疫疾患であるSLE（全身性エリテマトーデス）を増悪させるというAOPである。本AOPについては、これまでコーチとの2回の会議を経て、AOP案の修正を行っており、主な論点とその対応は以下の通りである。AOとしてのSLEの増悪について具体的な測定指標が明確になっていないという指摘については、ヒトやSLEモデルマウスでのglomerulonephritis（糸球体腎炎）の診断法（診断や剖検所見）を記載することを提案した。またAOに相当するin vitroの試験法は見いだせなかったため、その旨記載することとした。

「Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response」は、JAK3の阻害によりL-2産生が抑制され、最終的にTDARの阻害となるというAOPである。本AOPについては、これまでコーチと3回の会議を行い、AOP案の修正を行っている。主

な議論と対応を以下に示す。JAKおよびSTATには様々なファミリーがあり、その組み合わせも様々であるが、本AOPにおいてJAK3-STAT5に限定しているのはダイアグラムをシンプルにするためである。またストレッサーを特異性の高いものに限定しているのも、他のJAKおよびSTATファミリーを阻害するストレッサーを除くためである。さらに先行しているカルシニューリン阻害のAOPとAOをなるべく共通にしているのは、将来AOPネットワークを構築することを目指しているためである。

D. 考察

AOPの開発は最終化まで2回のレビュー（内部及び外部）を経る必要があり、平均約1000日を要すると言われる膨大で緻密な作業である。そこで新たにコーチ制が導入され、レビューの効率化が図られている。一方で、コーチあるいはレビュアーの負担の大きさから、EAGMSTでは将来的には独立したジャーナルでのレビューも検討されている。こうした流動的な状況においても、本研究では、1つが外部レビューに入り、3つがコーチによる指導を受けながらAOPの開発を進めている。

今回、多くの指摘を受けたのが、記載したストレッサーが少なく、行政的活用という観点から汎用性に欠けるのではないかというものである。AOPを作成する際には、そのメカニズムが量的関係も含めて詳細に解明されていることが必要であるが、その条件を満たしつつ汎用性を高めるには、今後Read acrossの適用や、AOPを共通のKEあるいはAOでつなぐ、いわゆるAOPネットワークの構築が必要となると考えられた。JAK3阻害に関するAOと、カルシニューリン阻害のAOを同じ「TDARの抑制」としたのは、将来のネットワーク化を想定したためであり、TLR刺激のAOPにIncreased Pro-inflammatory mediatorsというより一般的なKEを挿入することの検討も、他のAOPとネットワーク化するためである。

免疫毒性は極めて複雑な毒性であり、エビデンスに基づいたAOPを多数作り、それらをネットワーク化することで全体像を明確にすることが重要と考える。免疫毒性に関するAOPは未だ承認されたものはないが、日本が大きく貢献できるよう引き続き取り組んでいく。

E. 結論

今年度は開発中の4種のPのうち一つが外部レビューに進み、残りの3つもコーチとともに開発を進めた。

F. 研究発表

F.1. 論文発表

なし

F.2. 学会発表

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4. 足利太可雄: 呼吸器感作性物質評価に関

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H. 添付資料

1. AOP:154
   Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response
2. AOP:313
   Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease
3. AOP:314
   Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus
4. AOP:315
   Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response

G. 知的財産権の出願・登録状況

なし

AOP ID and Title:

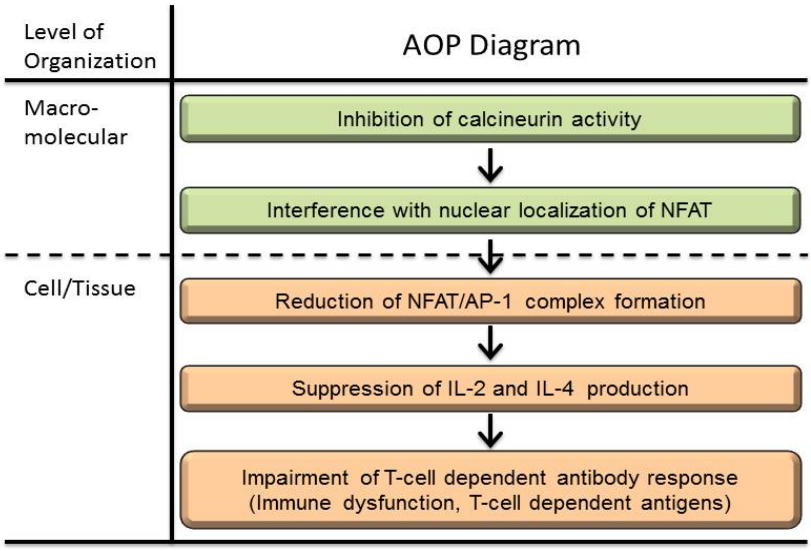
SNAPSHOT

Created at: 2020-05-18 09:20

AOP 154: Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response

Short Title: Immunosuppression

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Open for comment. Do not cite	EAGMST Under Review	1.38	Included in OECD Work Plan

Abstract

Calcineurin (CN), a protein phosphatase, is known to impair immune function when its phosphatase activation is inhibited. The relationship between CN and immune functions is well understood, and immunosuppressants that work by inhibiting CN have been developed.

CN inhibitors (CNIs) inhibit CN phosphatase activity to suppress many kinds of immune functions and have been used to prevent hyper immune reactions such as rejection and graft versus host disease (GVHD), and treat autoimmune and allergic disorders such as psoriasis and atopic dermatitis. On the other hand, CNIs are reported to induce immunosuppression-derived adverse effects such as increased frequency and/or severity of infections and increased tumor incidences. CNIs might affect kinds of T-cell derived immune functions to induce compromised

host. Among the affected immune functions. T-cell dependent antibody response (TDAR) is the important factor to resist infections and thought to be the useful endpoint on evaluating immunotoxicity of chemicals; therefore, this AOP describes the linkage between the inhibition of CN activity and impairment of TDAR.

CN activity is inhibited when stressors of CNIs bind to CN with their respective immunophilins, which interferes with the nuclear localization of nuclear factor of activated T cells (NFAT), a substrate of CN. As a result, the formation of functional NFAT complexes with activator protein-1 (AP-1) that bind at the site of IL-2, IL-4 and other T cell -derived cytokine promoters is reduced, thereby suppressing production of these cytokines. Among the affected cytokines from each of the helper T cell subsets, reduced production of IL-2 and IL-4 affects the proliferation and differentiation of B-cells to suppress TDAR.

We have identified a number of key events along this pathway and determined the key event relationships, based on which we have created an AOP for inhibition of CN activity leading to impaired TDAR.

Since CN expresses in cells among vast variety of species, this AOP might be applicable to many mammal species, including humans and rodents.

Background

Although there are stressors that inhibit CN activity, this AOP is mainly based on an understanding of immunosuppression caused by FK506 and FKBP12 complexes, on which a significant body of scientific literature has been published.

We look forward to future amendments to this AOP with up-to-date information on other stressors, which will more clarify the linkage between inhibition of CN activity and impairment of TDAR.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	980	Inhibition, Calcineurin Activity (https://aopwiki.org/events/980)	Inhibition, Calcineurin Activity
2	KE	979	Interference, nuclear localization of NFAT (https://aopwiki.org/events/979)	Interference, nuclear localization of NFAT
3	KE	981	Reduction, NFAT/AP-1 complex formation (https://aopwiki.org/events/981)	Reduction, NFAT/AP-1 complex formation
4	KE	1202	Suppression, IL-2 and IL-4 production (https://aopwiki.org/events/1202)	Suppression, IL-2 and IL-4 production
5	AO	984	Impairment, T-cell dependent antibody response (https://aopwiki.org/events/984)	Impairment, T-cell dependent antibody response

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition, Calcineurin Activity (https://aopwiki.org/relationships/1508)	adjacent	Interference, nuclear localization of NFAT	Moderate	Moderate
Interference, nuclear localization of NFAT (https://aopwiki.org/relationships/1017)	adjacent	Reduction, NFAT/AP-1 complex formation	High	High
Reduction, NFAT/AP-1 complex formation (https://aopwiki.org/relationships/1509)	adjacent	Suppression, IL-2 and IL-4 production	High	High

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Suppression, IL-2 and IL-4 production (https://aopwiki.org/relationships/1510)	adjacent	Impairment, T-cell dependent antibody response	High	High

Stressors

Name	Evidence
Tacrolimus	High
Cyclosporin	High
Pimecrolimus	High
Gossypol	Moderate
Kaempferol	Moderate
Dodecylbenzene sulfonate	Moderate
Dibefurin	Moderate
Ascomycin	Moderate
1,5-dibenzoyloxymethyl-norcantharidin	Moderate

Overall Assessment of the AOP

Inhibition of CN might induce suppression of cytokines production from all the T helper cell subsets as well as other immune functions of other immune cells. Suppression of cell-mediated immunity is involved in the pharmacology of preventing hyper immune reactions such as rejection and GVHD, and treatment of autoimmune and allergic disorders such as psoriasis and atopic dermatitis. On the other hand, CN inhibition might induce immunosuppression-derived adverse outcomes. One of the effects is increased frequency and/or severity of infections. Compromised host might be related with impairment of multiple immune functions; however, impaired TDAR deems to be usually related. Moreover, TDAR is the frequently used measurable endpoint in immunotoxicity testing according the ICH S8 or US EPA OPPTS 870.7800 immunotoxicity testing guideline. Therefore, the present AOP focus on CN inhibition-induced impairment of TDAR.

CN phosphatase activity is inhibited when stressors bind to Calcineurin-A (CnA) with immunophilins, which interferes with the nuclear localization of NFAT, a substrate of CN. As a result, the formation of functional NFAT/ AP-1 complexes that bind at the site of IL-2 , IL-4 and other cytokine promoters in each of the T helper cell subsets is reduced, thereby suppressing production of these cytokines. Among the affected cytokines TDAR is impaired mainly by the suppression of production of IL-2 and IL-4, which affect the proliferation and differentiation of B-cells to lower TDAR. We have identified a number of key events (KEs) along this pathway, and based on these key event relationships (KERs), created an AOP for inhibition of CN activity leading to impaired TDAR.

Since each KE involving MIE and AO is quantifiable, and shows similar dose responses with the CNIs in vitro, this AOP is useful for understanding immunosuppression due to inhibition of CN activity. In addition, each KER is based on sufficient scientific evidence and exhibits no contradiction with dose responses of adjacent KEs.

Since CN/NFAT system expresses in cells among vast variety of species and the function in immune system is common in at least human and mice, this AOP might be applicable to many mammalian species, including humans and rodents.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Macaca fascicularis	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Sex Applicability

Sex	Evidence
Mixed	High

The proposed AOP regarding inhibition of CN activity leading to impaired TDAR is not dependent on life stage, sex, or age. Since tacrolimus (FK506) ointment (Protopic) is approved for pediatric atopic dermatitis, the MOA for immunosuppression appears to be applicable to all life stages. The applicable state is considered supported by the draft FDA guidance for immunotoxicology that was recently issued (2020) indicating that “example of immunotoxicology testing could included TDAR assay” to address the concern of immunotoxicity in offspring in juvenile animal studies.

Since FK506 or Cyclosporine A (CsA)-induced outcomes in humans are mimicked by similar responses in a variety of animal models including non-human primates and rodents, immunosuppression induced by inhibition of CN activity is considered to occur across a variety of mammalian species.

In addition to the drugs, it is known that CN activity is suppressed by alkeylbenzene sulfonate (dodecylbenzene sulfonate) extracted from an acrylonitrile butadiene rubber (Ito et al. 2013)., suggesting that the proposed AOP would be applicable to non-pharmacological agents.

For the chemicals such as pesticide, TDAR is also recommended in the US EPA OPPTS 870.7800 immunotoxicity testing guideline.

Essentiality of the Key Events

Essentiality is supported by several knockout animals as follows.

Stage	Essentiality	Evidence	Supported by literatures
MIE and later	CnA-KO mice	Strong	The CN molecule consists of two regions, CnA and CnB, of which CnA exhibits phosphatase activity. In CnA-KO mice, T-cell proliferation in response to ovalbumin stimulation is lower than that for wild-type mice and is not complemented by normal antibody producing cells. In addition, when stimulated with ovalbumin, CnA-KO mice produce less IFN-γ, IL-2, and IL-4 than wild-type mice. However, primary antibody response in CnA-KO mice is normal in response to TNP-ovalbumin, which means that CnA deficiency affects only on T cell-dependent antibody response (TDAR) (Zhang et al. 1996).

KE1 and later	NFAT-KO mice	Strong	The following phenotypes are observed in NFAT-KO mice: moderate hyperproliferation with splenomegaly, moderately enhanced B- and T-cell responses, with bias towards Th2-cell response, decreased IFN-γ production in response to T-cell receptor (TCR) ligation, reduced proliferative responses by T cells, impaired repopulation of the thymus and lymphoid organs, impaired Th2- cell responses and IL-4 production, grossly impaired T-cell effector functions, profound defects in cytokine production and cytolytic activity, B-cell hyperactivity, impaired development of CD4 and CD8 single-positive cells, increased apoptosis of double-positive thymocytes, and mild hyperactivation of peripheral T cells. Therefore, the study of NFAT-KO mice shows that NFAT is involved in a wide range of immune responses, and some of these phenomenon are known to be regulated by CN. Suppression of T-cell-derived cytokines is noted both in CnA-knockout and NFAT-knockout mice, which indicates that the production of T-cell derived cytokines such as IL-2 and IL-4 is regulated by the CN-NFAT system (Macian, 2005).
Stressor	FKBP12-KO mice	Moderate	FK506 induces suppression of immune responses; however, there is no literature showing a relationship of a relationship between FKBP12 knockout and the immune system in the FKBP12-KO mouse model. Steric structure of FKBP12/FK506 complex is considered the key factor for inhibition of CN phosphatase activity, but not for the enzymatic activities of FKBP12.

Weight of Evidence Summary

Biological Plausibility

T-cell functions are mainly regulated by the CN-NFAT system and suppression of CN activity in T cells is known to induce multiple types of immunosuppression, including T cell-dependent antibody response (TDAR).

Experiments with T cells indicate that TCR stimulation brings about increases in intracellular concentrations of Ca2+ that trigger CN activity, thereby inducing nuclear localization of substrate NFAT per dephosphorylation. The localized NFAT forms complexes with activator protein 1 (AP-1) at the promoter sites of the T-cell cytokine genes and induces production of the cytokines.

CN phosphatase activity is known to be inhibited by the formation of immunophilin-CN inhibitor (CNI) complexes, such as CsA/cyclophilin complexes or FK506/FK506-binding protein (FKBP) 12 complexes. Immunophilins are a general class of proteins that exhibit peptidyl-propyl isomerase (PPIase) activity, but there is no commonality between amino-acid sequences of the two classes of immunophilins. The three-dimensional structures of immunophilin complexes are essential to the inhibition of CN phosphatase activity, even though their enzymatic activities are not.

It is also known that one of the effects on immune function when CNI forms complexes with its respective immunophilin and inhibits CN activity is the suppression of IL-2 and other T-cell derived cytokine production. It is further known that inhibition of CN leads to suppression of TDAR because IL-2 and IL-4 mainly promote the proliferation, class switching, differentiation, and maturation of B-cells.

Furthermore, CN-NFAT also exists in B-cells and it has been reported that CNIs do suppress production of certain cytokines from them. At the time of our review of the literature, however, we did not find any reports of a direct effect of CN inhibition on B-cells, such as changes in proliferation, class switching, differentiation, or maturation of B-cells.

Also, although CN-NFAT is known to exist in dendritic cells, natural killer T (NKT) cells, and other types of cells in which it regulates the expression of IL-2 receptors, there are no reports of effects on the production of T cell-dependent antibodies due to CNI-induced alteration in expression of IL-2 receptors in these cells.

CN-NFAT system-mediated immunosuppression is well understood based on the pharmacology of some CNI drugs; therefore, AOP of CN inhibition-induced suppression of TDAR is useful for prediction of CN-mediated immunotoxicity.

KER	KE_{up}-KE_{down}	Evidence	Rationales supported by literatures

KER1	CN inhibition to interference, NFAT nuclear translocation	Moderate	CN phosphatase activation through TCR stimulation dephosphorylates NFAT, thereby promoting nuclear localization of NFAT. CN phosphatase activity in T cells could be inhibited by CNI/immunophilin complexes, thus interfering with dephosphorylation and nuclear localization of NFAT. The known mechanisms for inhibition of CN phosphatase activity by FK506, CsA, or other CNIs are initiated by the formation of complexes with their respective immunophilin species. Immunophilins are general classes of proteins that exhibit PPIase activity, but modification of these functions is unrelated to inhibition of CN activity and thus thought to arise in the molecular structure of the complexes (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).
KER2	Interference, nuclear localization NFAT to reduction, NFAT/AP-1 complex formation	Strong	CN activity dephosphorylates NFAT, thereby promoting its nuclear translocation. Nuclear-located NFAT binds with AP-1 at the promoter regions of the cytokine genes to promote T-cell cytokine production. Inhibition of dephosphorylation of NFAT by CNIs prevents nuclear localization of NFAT and resultant binding with AP-1 at the promoter region of the T cell cytokine genes. NFAT has NLS and NES among and adjacent to the N-terminal region rich in SP motifs, and once the SP region is dephosphorylated, the NLS is exposed and the NES is covered, which leads to translocation of NFAT into the nucleus (Matsuda and Koyasu 2000). CNIs interference with the nuclear localization of NFAT in T cells leads to a reduction in the formation of NFAT/AP-1 complexes, thereby suppressing transcription of IL-2, IL-4, and a number of other cytokines (Maguire et al. 2013, Jain et al. 1992, Jain et al. 1993).
KER3	Reduction, NFAT/AP-1 complex formation to suppression of IL-2 and IL-4 production	Strong	NFAT/AP-1 complexes bind to the promoter regions of the cytokine genes, which promotes production of cytokines from T cells. Of these cytokines, IL-2 and IL-4 have a major role in promoting proliferation, maturation and class-switching of B cells, and development of TDAR. Reduction of NFAT/AP-1 complex formation in the nucleus due to inhibition CN activity by CNIs suppresses production of T-cell derived cytokines, including IL-2 and IL-4. T-5224, a selective c-Fos/AP-1 inhibitor, inhibits the DNA-binding activity of AP-1 in primary murine T cells. T-5224 also inhibits CD25 (one of IL-2 receptors) up-regulation, IL-2 production, and c-Fos DNA-binding activity in mice (Yoshida et al. 2015). Dexamethasone represses the IL-2 mRNA induction. glucocorticoid-induced leucine zipper (GILZ) is one of the most prominent glucocorticoid-induced genes, and inhibited the induction of the NFAT reporter and interferes with the AP-1 component of the NFAT/AP-1 complex. GILZ also inhibits the IL-2 promoter (Mittelstadt et al. 2001). Ursolic acid suppressed activation of three immunoregulatory transcription factors NF-kB, NFAT and AP-1. Treatment of lymphocytes and CD4+ T cells with ursolic acid inhibited secretion of IL-2 and IL-4 cytokines. Treatment of CD4+ T cells with ursolic acid suppressed mRNA level of IL-2. Treatment of lymphocytes with ursolic acid inhibited the upregulation of CD25 expression on T cells (Checker et al. 2012).

KER4	Suppression of IL-2 and IL-4 production to impaired TDAR	Strong	T cell-derived cytokines play important roles in TDAR. Among them, IL-2 promotes proliferation of B cells, and IL-4 affects maturation and class switching of B cells as well as proliferation. Inhibition of CN activity by CNIs is known to suppress production of multiple cytokine species from T cells. Of these cytokines and receptors, suppression of IL-2 and IL-4 production mainly leads to impairment of TDAR. Suppressed production of other cytokines due to inhibition of CN activity exhibits only minor effects, if any, on TDAR. CsA is known one of the calcineurin inhibitiors. CsA-treatment is reported to suppresses the productions of IL-2 and IL-4 and result in the reduction of the productions of antigen-specific IgM and IgG in cynomolgus monkeys (Gaida K. 2015). Dupilumab is known as anti-IL-4/13 receptor (IL-4/13R) antibody. Dupilumab (Dupixent) reduces productions of immunoglobulin (Ig) E and antigen specific IgG1 in mice (Sanofi K.K. 2018). It suggests that the blocking of IL-4 signaling by anti-IL-4/13R antibody results in the decrease in T cell dependent antibody production. Th2 cell produces cytokines including IL-4. Suplatast tosilate (IPD) is known as an inhibitor of the production of IL-4 and IL-5 from Th2 cells and reduces the production of antigen specific IgE in human cell culture and mice (Taiho Pharmaceutical 2013). These findings suggests that the reduction of IL-4 production by the inhibitor of Th2 cell cytokines results in reduced production of IgE and/or IgG1 through inhibitions of maturation, proliferation and class switching of B cells. IL-2 binds to IL-2 receptor (IL-2R) and acts on T cell. CD25 is one of IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016). FK506 and CsA suppress mRNA expression levels of cytokines in T cells including IL-2 and IL-4 that stimulate proliferation of B cells as well as B cell activation and class switching (Heidt et al, 2010).

Empirical Support

KER	KE_{up}-KE_{down}	Evidence	Empirical support of KERs
KER1	Inhibition, calcineurin activity leads to interference, nuclear localization of NFAT	Moderate	CN phosphatase activity is inhibited by CNI of FK506 with IC50 values of 0.5 nM (FK506) and 5nM (CsA) after 1 hours treatment (Fruman et al.1992). Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the concentration from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 µM (1000 nM) under the conditions of 2 hours treatment of tacrolimus (Maguire et al. 2013). Interference with translocation of NFAT to the nucleus is also detected using gel mobility shift assay to test nuclear extracts and cytoplasmic extracts, in which the examined concentration of FK506 was 10ng/mL (Flanagan et al. 1991). CN phosphatase activity and nuclear translocation of NFAT seems to be suppressed by CNIs at the similar ranges of doses and reaction times of 1 to 2 hours.

KER2	Interference, nuclear localization of NFAT leads to reduction, NFAT/AP-1 complex formation	Strong	Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the concentration from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 µM (1000 nM) under the conditions of 2 hours treatment (Maguire et al. 2013). Treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders the formation of functional NFAT/AP-1 in the nucleus (Flanagan et al. 1991). The experiment of gel mobility shift assay using Ar-5 human T cells stimulated with cross-linked anti-CD3 antibody showed that NFAT/AP-1 (cFos and Jun) complexes were found only in the nuclear extract with preexisting NFAT in the cytoplasm after T cell stimulation and that the NFAT/AP-1 complexes in the nucleus decreased after 2 hours treatment with CsA at 1µM (Jain et al. 1992). Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991). NFAT/AP-1 complex formation was also reported to be inhibited by CNI (Rao et al. 1997). Quantitative data on NFAT/AP-1 complex formation in the nucleus is insufficient; however, inhibition of nuclear localization of NFAT and following NFAT/AP-1 complex formation in the nucleus are simultaneously detected by gel mobility shift assay at the concentration of FK506 within the range for inhibition of nuclear translocation of NFAT using imaging flowcytometry after 2 hours culture of T cells.

KER3	Reduction, NFAT/AP-1 complex formation leads to suppression, IL-2 and IL-4 production	Moderate	In NFATp- and NFAT4-deficient mice, cultured splenocytes bound anti-CD3 for 48 h indicates decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998). In purified T cell from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 and CD25 (one of IL-2 receptors) up-regulation at 80 µg/mL, and IL-2 production in a dose-dependent manner from 40 to 80 µg/mL (Yoshida et al. 2015). In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 µM for 4 h. Secretion of IL-2 and IL-4 was inhibited in lymphocytes stimulated with concanavalin A for 24 h at concentrations of 0.5, 1 and 5 µM of ursolic acid, and lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h at concentration of 5 µM of ursolic acid. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 µM for 4 h. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 µM for 4 h (Checker et al. 2012). Gel mobility shift assay revealed that treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders NFAT nuclear translocation and following formation of NFAT/AP-1 complexes in the nucleus (Flanagan et al. 1991). Preceding NFAT nuclear localization after T cell activation is suppressed with FK506 at the dose range of 0.01nM (Jarkat T cells) or 10nM (CD4+ T cells) to 1µM (Maguire et al. 2013), and NFAT nuclear localization and NFAT/AP-1 complex formation is shown to be strongly related (Jain et al. 1992, Jain et al. 1993). In CD3/PMA-activated human T cells, FK506 suppressed production of IL-2, IL-4, and IFN-γ at the concentrations of 1.2 to 12.5 nM after 22 to 24 hours culture as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA in a dose-dependent (10 nM) manner (Dumont et al. 1998). Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC25 and IC50 for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC25 and IC50 for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018). In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010). Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014). In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) reduced IL-2 release in response to keyhole limpet hemocyanine (Alessandro, B. et al. 2003). Therefore, concentration of CNI needed for inhibition of NFAT/AP-1 complex formation in the nucleus is higher than that for inhibition of IL-2 and IL-4 production.Time lag is found between the two KEs; 2 hours for KE2 and 22 to 48 hours for KE3.

KER4	Suppression, IL-2 and IL-4 production leads to Impairment, T-cell dependent antibody response	Strong	Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015). In the allergen-induced pneumonia model in mice, dupilumab (anti-IL-4/13R antibody) reduced productions of IgE and antigen specific IgG1 at 25 mg/kg of twice weekly subcutaneous administration for 4weeks (Sanofi K.K. 2018). In mice immunized with dinitrophenyl antigen by i.p. injection, suplatast tosilate (an inhibitor of the production of cytokines such as IL-4 and IL-5 on Th2 cell) reduced productions of antigen specific IgE at 10, 20, 50 and 100 mg/kg of oral administration for 5 days (Taiho Pharmaceutical 2013). In human cell culture immunized with Japanese cedar antigen, suplatast tosilate reduced productions of antigen specific IgE at the concentration of 10 µg/mL for 10 days (Taiho Pharmaceutical 2013). 1,2:5,6-dibenzanthracene single adoministration suppressed production of IL-2 and total IgG antibody in mice at the dose levels of 3 and 30 mg/kg(Donna, C. et al. 2010). In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) for 21 days reduced IL-2 release in response to KLH and decrease in anti-KLH IgG(Alessandro, B. et al. 2003). FK506 or CsA suppressed production of IL-2 in mouse mixed lymphocyte reaction (MLR) at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA as well as in human MLR at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA (Kino et al. 1987a). In CD3/phorbol 12-myristate-13-acetate-activated human T cells, FK506 suppressed production of IL-2, IL-4 and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM as well as inhibited expression of IL-2, IL-4 and IFN-γ mRNA at the concentrations of 10 nM. (Dumont et al. 1998). Rats were treated with FK506 for over four weeks and immunized with keyhole limpet hemocyanine (KLH), after which serum concentration of anti-KLH IgM and IgG reduced at the dose levels of 3 mg/kg/day (Ulrich et al. 2004). Mice were treated with FK506 or CsA for 4 days, and immunized with sheep red blood cells (SRBC), after which antigen-specific plaque-forming splenocytes reduced at the dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b). After 9-day culture of B cells and non-pre-activated T cell stimulationwith FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41 and 83nM) of CsA (Heidt et al, 2010). After 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at the concentrations of 0.01 to 100 ng/mL (0.01 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.08 to 832 nM) of CsA (Sakuma et al, 2001). In vitro suppression of T-cell–derived cytokines and T-cell–dependent antibody production or antibody production after polyclonal T-cell stimulation showed similar dose responses to CNIs. Time gaps were found, however, between these two events, which showed earlier onset of cytokine production and delayed onset of antibody production.

Based on these findings of empirical support, each KE involving MIE and AO except for KE2 shows similar dose responses to the CNIs in vitro; however, culture time lag is noted, in that, 1 hour for MIE, 2 hours for KE1 and KE2, 22 to 24 hours for KE3 and more than days for AO.

Quantitative Consideration

KER1

There have been no literature available to show clear quantitative relationship between the inhibition of CN phosphatase activity and nuclear translocation of NFAT; however, the dose responses of CN phosphatase activity and nuclear translocation of NFAT to CNI deem to be the same.

KER2:

Gel mobility shift assay of activated T cells showed that NFAT/AP-1 complexes are only found in nuclear extract, which indicates a strong relationship between the nuclear translocation of NFAT and simultaneous complex formation with AP-1 in the nucleus. CNI treatment clearly suppresses the complex formation of nuclear located NFAT and AP-1 in the nucleus, which also shows the solid relationship between these adjacent two KEs although quantitative data on suppressed NFAT/AP-1 complex formation is insufficient (Flaganan W.M. et al. 1991).

KER3:

The quantitative relationship between the decreased formation of NFAT/AP-1 complexes and the production of IL2/IL-4 formation induced by CNIs has not been reported.

However, as mentioned in the empirical support, nuclear localization of NFAT is strongly related to NFAT/AP-1 complex formation in the nucleus based on the fact that these two events are detected simultaneously by gel mobility shift assay, and the dose responses of IL2/IL-4 production and nuclear translocation of NFAT inhibited by CNI are similar; therefore, dose ranges of CNI in the inhibitions of IL2/IL-4 production and NFAT/AP-1 complex formation in the nucleus might also be the same.

In addition, T-5224 and ursolic acid inhibit AP-1 DNA binding activity or production of NF-κB, NFAT and AP-1, respectively, and both suppress the IL-2 and/or IL-4 production with dose dependent manner including the doses of inhibiting NFAT-AP-1 system (Yoshida et al. 2015, Checker et al. 2012).

KER4:

Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015).

Inhibition of IL-4 production in mice treated with oral administration of suplatast tosilate suppresses antigen-specific IgE production with a dose-dependent manner (Taiho Pharmaceutical 2013). In the inhibition of IL-4 production in human cell culture by suplatast tosilate at the concentration of 10 µg/mL for 10 days, antigen specific IgE production was suppressed from 56 to 72% and IL-4 production was suppressed from 58 to 76% (Taiho Pharmaceutical 2013).

As for IL-2 and antibody production, in vitro T-cell-induced polyclonal B cell activation to produce antibody was inhibited with anti-IL-2 and anti-IL-2R antibodies. T (Owens T, 1991).

In the human T-B cell co-culture stimulated with anti-CD3 monoclonal antibody, CNIs of FK506 and CsA lowered the levels of T-cell cytokines including IL-2 and IL-4 and inhibited IgM and IgG productions with a dose-dependent manner (Heidt S. 2010).

These results show the quantitative relationships between the inhibition of IL-4 or IL-2 by specific antibodies or CNI and suppression of antibody production.

Considerations for Potential Applications of the AOP (optional)

CN is expressed in T cells as well as other types of immune cells like B cells and dendritic cells. CNIs suppress many kinds of immune functions leading to increased susceptibility to infections and decreased hyper immune reactions such as rejection and GVHD. Among these, TDAR is considered to be the important endpoint of immunotoxicity, because T cells, B cells, and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR. Thus, changes in any of these immune cell populations can influence TDAR.

Moreover, when evaluating the immunotoxicity of pharmaceuticals, the ICH S8 immunotoxicity testing guideline recommends that TDAR be evaluated whenever the target cells of immunotoxicity are not clear based on pharmacology and findings in standard toxicity studies.

The draft FDA guidance of nonclinical safety evaluation for immunotoxicology is recently issued (2020) and recommends TDAR assay. Because TDAR is a common secondary assay that requires functionality of several key immune cell subtypes (e.g., antigen-presenting cells, T-helper cells, B cells).

For the assessment for pesticides, US EPA OPPTS 870.7800 immunotoxicity testing guideline recommends TDAR using sheep red blood cells.

As a part of an IATA of immunotoxicology, the present AOP could be used to predict whether or not a compound that potentially acts on T cells could also affect TDAR. On the other hand, it would be inappropriate to use the present AOP alone as an alternative to TDAR measurement in the ICH S8 or US EPA OPPTS 870.7800 immunotoxicity testing guideline.

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Appendix 1

List of MIEs in this AOP

Event: 980: Inhibition, Calcineurin Activity (https://aopwiki.org/events/980)

Short Name: Inhibition, Calcineurin Activity

Key Event Component

Process	Object	Action
binding	FK506-binding protein 15	increased
binding	FKBP12 (Arabidopsis thaliana)	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	MolecularInitiatingEvent

Stressors

Name
Tacrorimus
Cyclosporin
Pimecrolimus
Dodecylbenzene sulfonate
Dibefurin
Gossypol
Ascomycin
Kaempferol
1,5-dibenzoyloxymethyl-norcantharidin

Biological Context

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

CN inhibitory activities (IC50) are shown in follows.

Tacrorimus: 0.4nM

Cyclosporin: 7nM

Pimecrolimus: 0.4 nM

Dodecylbenzene sulfonate 9.3 uM

Dibefurin: 44 uM

Gossypol: 17 uM

Ascomycin: 0.7 nM

1,5-dibenzoyloxymethyl-norcantharidin: 7 uM

Kaempferol: 51.3 uM

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus rattus	Rattus rattus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10117)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

CN is broadly distributed in T-cells, B-cells, and throughout the body. The structure of CnA and CnB is highly conserved from yeasts to humans. Also highly conserved are the amino acid sequences of the catalytic and regulatory domains of CnA isoforms from different organisms (Kincaid. 1996).

As for immunophilins, of which complexes inhibit the CN activity, FKBP is found in a wide variety of organisms, from prokaryotes to multicellular organisms (Siekierka et al. 1989a). Multiple subfamilies of FKBP have been reported, with at least eight types having been found in mammals. FKBP12 is reported to be expressed in B-cells, Langerhans cells and mast cells as well as in T-cells of humans, mice and other mammalian species.

Cyclophilins have been found in mammals, plants, insects, fungi and bacteria. They are structurally conserved throughout evolution and all living beings have PPIase activity (Wang P et al. 2005).

However, inhibition of CN phosphatase activity through immunophilin-CNI complex has been reported at least in rodents and humans.

Key Event Description

Calcineurin (CN) is a heterodimer that comprises a catalytic subunit (CnA), which handles phosphatase activity as well as calmodulin binding, and a Ca-binding regulatory subunit (CnB), which regulates intracellular calcium as well as CnA (Klee et al. 1988, Zhang et al. 1996). CnA, a 59kDa protein, has a serine-threonine phosphatase domain.

Immunophilins are a general class of proteins that exhibit peptidyl-propyl isomerase (PPIase) activity (Barik. 2006) and an immunophilin-CN inhibitor (CNI) complex such as FKBP12- FK506 and cyclophilin-CsA binds directly to CnA in the cell, causing steric hindrance of substrate binding to CN, which inhibits the phosphatase activity of CN without any contribution of PPIase activity (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

How it is Measured or Detected

Phosphatase activity can be measured using a phosphatase assay. CN, calmodulin, FK506, and FKBP are incubated together, and the phosphatase activity is measured at various concentrations of FKBP. Kinetic analysis of FKBP12 concentration-dependent phosphatase activity and calculation of Ki inhibition of CN by the FKBP12-FK506 complex are conducted. (Bram et al. 1993). Phosphatase activity of CN in the presence of cyclosporin A (CsA) , gossypol or dibefurin can also be determined in the similar manner (Sieber et al. 2009).

Immunophilin-CNI complexes directly inhibit phosphatase activity of CN, therefore, as a surrogate measurement of the CN activity, the binding of CsA with cyclophilin can be detected using an ELISA kit. Microtiter plates precoated with BSA and conjugated to cyclosporin are incubated with cyclophilin. Bound cyclophilin is then revealed by incubation with anti-cyclophilin rabbit antiserum followed by incubation with anti-rabbit globulin goat IgG coupled to alkaline phosphatase (Quesniaux et al. 1987).

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List of Key Events in the AOP

Event: 979: Interference, nuclear localization of NFAT (https://aopwiki.org/events/979)

Short Name: Interference, nuclear localization of NFAT

Key Event Component

Process	Object	Action
genetic interference	NFAT protein	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	KeyEvent

Stressors

Name
Tacrolimus
Cyclosporin

Biological Context

Level of Biological Organization
Molecular

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents and other mammalian species (Rao et al. 1997).

Key Event Description

The nuclear factor of activated T cells (NFAT) is a substrate of calcineurin (CN) (Rao et al. 1997). A NFAT has an N-terminal with a plurality of SP motifs rich in serine and proline, which are controlled by means of phosphorylation and dephosphorylation. There is a nuclear localization signal (NLS) held between these SP regions as well as a nuclear export signal (NES) in the N-terminal adjacent to the SP motifs (Beals et al. 1997, Zhu and McKeon 1999, Serfling et al. 2000). SP motifs ordinarily are phosphorylated, which covers the NLS and leaves the NES exposed, so that NFAT localizes in cytoplasm. When SP motifs are dephosphorylated by activated CN, the NLS is exposed and the NES is covered, thereby promoting nuclear localization of NFAT (Matsuda and Koyasu 2000, Zhu and McKeon 1999). When T-cell activation takes place, T-cell–receptor-mediated stimulus increases the intracellular concentration of calcium and activates a regulatory subunit (CnB), which subsequently induces a catalytic subunit (CnA) phosphatase activation, leading to dephosphorylation of NFAT followed by nuclear localization. CNI-immunophilin complexes inhibit CN phosphatase activation, thereby interfering with NFAT nuclear localization (Bhattacharyya et al.2011).

Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the concentration from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 µM (1000 nM) under the conditions of 2 hours treatment (Maguire et al. 2013).

How it is Measured or Detected

Nuclear translocation of NFAT can be tested by imaging flowcytometer, in which lymphocytes are treated with fluorescence-labeled anti-NFAT antibody and DAPI (nuclear stain) and intracellular distribution of NFAT is analyzed by imaging flowcytometry with image analysis (Maguire O et al. 2013).

Interference with translocation of NFAT to the nucleus can be detected using a gel mobility shift assay of nuclear or cytoplasmic extracts electrophoresed with end-labeled NFAT-binding site from human IL-2 enhancer (Flanagan et al. 1991).

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Event: 981: Reduction, NFAT/AP-1 complex formation (https://aopwiki.org/events/981)

Short Name: Reduction, NFAT/AP-1 complex formation

Key Event Component

Process	Object	Action
cytokine production involved in inflammatory response	NFAT activation molecule 1	decreased
cell activation		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	KeyEvent

Stressors

Name
Tacrolimus
Cyclosporin

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
T cell

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

CN-NFAT system functionality is common among mammalian species, including humans and rodents. It is also possible that FK506-induced interference with NFAT/AP-1 complex formation at the promoter site of the IL-2 gene is common among mammalian T cells, including those of humans and rodents (Flanagan et al. 1991).

Key Event Description

Activated nuclear factor of activated T cells (NFAT) that has localized to the nucleus binds cooperatively at the site of the Interleukin-2 (IL-2) promoter with activator protein-1 (AP-1), which is a heterodimer comprising a Fos and a Jun protein (Schreiber and Crabtree 1992, Jain et al. 1992), thereby inducing transcription of IL-2 (Jain et al. 1993). Interfered nuclear localization of NFAT, induced by FK506, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991).

NFAT is known to bind cooperatively at the promoters of Interleukin-4 (IL-4) and other T-cell cytokines as well as that of IL-2 (Macian et al. 2005).

Treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders the formation of functional NFAT/AP-1 in the nucleus (Flanagan et al. 1991).

How it is Measured or Detected

Reductions in NFAT/AP-1 complex formation can be detected using a gel shift assay to test nuclear extracts from either stimulated or unstimulated Ar-5 T cells with radio-labelled NFAT binding oligonucleotide from murine IL-2 promoter. Anti-Fos and anti-Jun antibodies are used to examine NFAT/AP-1 complex formation (Jain et al. 1992).

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Event: 1202: Suppression, IL-2 and IL-4 production (https://aopwiki.org/events/1202)

Short Name: Suppression, IL-2 and IL-4 production

Key Event Component

Process	Object	Action
interleukin-2 production	interleukin-2	decreased

Process	Object	Action
interleukin-4 production	interleukin-4	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	KeyEvent

Stressors

Name
Tacrolimus
Cyclosporin
Dexamethasone
Azathioprine
Methotrexate
Benzo(a)pyrene
Urethane
1,2:5,6-dibenzanthracene
psychosocial stress

Biological Context

Level of Biological Organization
Cellular

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
cynomolgus monkey	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

CNIs suppress production of IL-2, IL-3, IL-4, IL-5, IFN-γ, Granulocyte Macrophage colony-stimulating Factor (GM-CSF), and other cytokines, as induced by CD2/CD3 or CD3/CD26 stimulation, in human peripheral blood mononuclear cells (PBMC) (Sakuma et al. 2001a). Also, CNIs (FK506 and CsA) suppress production of IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, Tumor Necrosis Factor-α, IFN-γ, and GM-CSF, as induced by CD3/PMA stimulation, in human PBMC (Dumont et al. 1998).

CNIs (FK506 and CsA) exhibit suppression of IL-2 production induced from mixed lymphocyte reactions in mice and humans (Kino, T et al. 1987a).

Treatment with CsA or 2C1.1 resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).

These facts indicate that Calcineurin-NFAT system-mediated suppression of cytokines is commonly found in humans, monkey and mice.

Key Event Description

Production of T cell cytokines including Interleukin (IL)-2 and IL-4 is regulated by nuclear factor of activated T cells (NFAT)/ activator protein-1 (AP-1) complexes. Activated NFAT/AP-1 complex that bind at the site of the IL-2 and IL-4 promoters, thereby induces transcription of IL-2 (Jain et al. 1993). For IL-2, NFAT proteins are necessary for IL-2 gene expression and cooperation of NFAT with AP-1 is required for IL-2 gene transcription. For IL-4, At least five different NFAT sites have been described in the IL-4 promoter with at least three of them being composite sites binding NFAT and AP-1 (Macián et al. 2001).

IL-2 binds to IL-2 receptor (IL-2R) and acts on T cell. CD25 is one of IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016).

Calcineurin inhibitors (CNIs) such as FK506 and cyclosporin A (CsA) hinder the formation of the functional NFAT/AP-1 complexes by interfering with NFAT nuclear localization (Flanagan et al. 1991). Reduced binding of NFAT/AP-1 complexes at the promoter site of the IL-2 gene lowers the transcription of the mRNA of IL-2 and the following cytokine production.

Transcription of IL-4 is also inhibited by CNIs in the same manner as IL-2 (Dumont et al. 1998).

In CD3/ phorbol 12-myristate-13-acetate (PMA)-activated human T cells, FK506 suppressed production of IL-2, IL-4, and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM after 22 to 24 hours culture as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA in a dose-dependent (10 nM) manner (Dumont et al. 1998).

Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC_{25} and IC_{50} for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC_{25} and IC_{50} for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018).

In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) reduced IL-2 release in response to keyhole limpet hemocyanine (KLH) (Alessandro, B. et al. 2003).

In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010).

Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).

CNIs is considered to increase carcinogenicity through the suppression of IL-2 and IL-4 production.

- Renal transplant patients on immunosuppressive therapy were found to develop cancer within 10 years after surgery (Luster, M.I. et al. 1993).

In experimental animal studies, carcinogenicity of FK506 was reported as follows.

- In mice subjected to topical application testing, in which 100 µL of FK506 ointment was applied once daily for two years to roughly 40% of the total body area, an increased incidence of lymphoma was found in mice of the 0.1% ointment group showing high blood concentrations of the drug (Maruho Co., Ltd 2014).
- In hairless albino mice, virtually all of which developed skin tumors after a 40-week exposure to ultraviolet light, application of a 1% FK506 ointment reduced the time to outbreak of the skin tumors. (Maruho Co., Ltd 2014).

How it is Measured or Detected

Quantitation of cytokine content was done on appropriately diluted samples, run in duplicate, using Sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) kits to test matched Antibody pairs with biotin-horseradish peroxidase-streptavidin detection and 3,3',5,5'-tetramethylbenzidine

substrate. ELISA plates were scanned in a Molecular Devices UVmax plate reader (Menlo Park, CA), using SOFT max software (Molecular Devices) (Dumont et al. 1998).

Ex vivo whole blood stimulated cytokine (IL-2, IL-4, IL-5, and IL-17) production assay in the supernatants were determined using an electrochemiluminescent immunoassay from Meso Scale Discovery (MSD; Gaithersburg, MD) (Kevin, G. et al. 2014).

Total RNA was extracted using RNeasy mini kit (Qiagen, Chatsworth, CA) and quantitated by absorbance at 260 nm. Cytokine mRNAs were detected using a RiboQuant MultiProbe RPA system (PharMingen, San Diego, CA). Riboprobes were 32P-labeled and hybridized overnight with 10 to 30 mg of the RNA samples. The hybridized RNA was treated with RNase and purified according to the RiboQuant protocol. The samples were then electrophoresed in 6% polyacrylamide-Tris-borate-EDTA-urea gels using the Seqi -Gen GT Nucleic Acid Electrophoresis Cell (Bio-Rad, Hercules, CA), or minigels (Novex, San Diego, CA). The gels were dried, exposed and quantitated in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using the ImageQuant software (Dumont et al. 1998).

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List of Adverse Outcomes in this AOP

Event: 984: Impairment, T-cell dependent antibody response (https://aopwiki.org/events/984)

Short Name: Impairment, T-cell dependent antibody response

Key Event Component

Process	Object	Action
Immunosuppression		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	AdverseOutcome

Stressors

Name
Tacrolimus
Cyclosporin
1,2:5,6-dibenzanthracene
psychosocial stress

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
cynomolgus monkey	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

CNIs induced impairment of TDAR is demonstrated with rodent studies. That is, oral administration of FK506 or CsA to mice for 4 days impaired the response of PFC in splenocytes after intravenous immunization with sheep erythrocytes (Kino et al. 1987). Likewise, oral administration of FK506 to rats over a four-week period reduced production of both anti-KLH-IgG and IgM antibodies after subcutaneous immunization with KLH (Ulrich et al. 2004). Moreover, Treatment with CsA at 50 mg/kg BID via oral gavage in cynomolgus monkey resulted in reduction of serum SRBC-specific IgM and IgG (Kevin, G. et al. 2014). As for humans, in vitro experiments showed that treatment with FK506 or CsA of peripheral blood mononuclear cells from blood-bank donors suppressed the production of IgM and IgG antibodies specific to T-cell–dependent antigens. (Heidt et al, 2009) Also, in SKW6.4 cells (IL-6–dependent, IgM-secreting, human B-cell line) cultures, FK506 or CsA suppressed the production of IgM antibodies in the presence of T-cell activation. (Sakuma et al. 2001b) Considering that FKF506 and CsA reduce T cell-derived cytokines including IL-2 and IL-4, these findings strongly suggest that impairment of TDAR following reduced production of such cytokines occurs at least in common among humans monkey and rodents.

Key Event Description

Antibody production to T-cell–dependent antigens is established through the coordination of B cells, antigen-presenting cells as well as T-cell–derived cytokines, which stimulate B cells to proliferate and differentiate. T-cell–dependent antibody response (TDAR) might be altered if any of these cell populations is affected.

Interleukin (IL)-2 stimulates B cells to proliferate through surface IL-2 receptors. IL-4 stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. Suppressing the production of these B-cell–related cytokines appears to impaire

TDAR, as seen in the result of FK506 treatment (Heidt et al, 2009).

IL-2 and IL-4 are produced and secreted by helper T cells and play important roles in the development of TDAR. IL-4 affects maturation and class switching of B cells as well as proliferation, both of which induces/enhances T cell dependent antibody production. IL-2 promotes differentiation of B cells through IL-2 stimulates differentiation of the activated T cell into T cell called Th2 cell. Therefore, suppressed production of IL-2 and IL-4 impairs TDAR (Alberts et al. 2008).

In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) decrease in anti- keyhole limpet hemocyanine (KLH) immunoglobulin (Ig)G. (Alessandro, B. et al. 2003).

In female B6C3F1 mice, 1,2:5,6-dibenzanthracene (DBA) exposure reduced total IgG antibody in spleen cell culture supernatants after in vitro stimulation with lipopolysaccharide (LPS) (Donna, C. et al. 2010).

Treatment with cyclosporin A (CsA) at 50 mg/kg BID via oral gavage in cynomolgus monkey resulted in reduction of serum sheep red blood cells (SRBC)-specific IgM and IgG (Kevin, G. et al. 2014).

After a 9-day culture of B cells and non-pre-activated T cell stimulation with FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41.6 and 83.2 nM) of CsA (Heidt et al, 2009).

After a 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated peripheral blood mononuclear cells (PBMC) culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at concentrations of 0.01 to 100 ng/mL (0.012 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.083 to 83.2 nM) of CsA (Sakuma et al. 2001b).

Rats were treated with FK506 for over four weeks and immunized with KLH, after which serum concentration of anti-KLH IgM and IgG was reduced at the dose level of 3 mg/kg/day (Ulrich et al. 2004).

Mice were treated with FK506 or CsA for 4 days, and immunized with SRBC, after which antigen-specific plaque-forming splenocytes were reduced at dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b).

As immunosuppression-derived adverse outcomes by calcineurin inhibition, FK506 and CsA increase the frequency and/or severity of infections and allergic reactions impaired TDAR deems to be one of the causative factors for these side effects . Some clinical trials of FK506 and CsA revealed these adverse effects as follows.

- In clinical trials of renal transplantation using FK506 or CsA, opportunistic infections such as candida, cytomegalovirus and herpes simplex virus were reported (Ekberg et al. 2007).
- In recipients of liver transplants treated with FK506 or CsA, opportunistic infections such as cytomegalovirus, hepatitis C virus, hepatitis B and herpes simplex virus were reported (Fung et al. 1991).
- Cardiac transplant patients treated with cyclosporin developed pulmonary infections within the first year after surgery (Luster, M.I. et al. 1993).
- In patients of X-linked autoimmune enteropathy treated with CsA or FK506, serum levels of IgE developed extremely high during the immunosuppressive therapy (Kawamura et al. 1997).
- Renal transplant recipients treated with belatacepy/mycophenolate (MMF)/predonisone or FK506/MMF/prednisone showed significantly lower the geometric mean hemagglutination inhibition titer against influenza vaccine, hemagglutination-specific IgG and isotype IgG1 antibodies, and IgG-antibody secreting cells response (Gangappa et al. 2019).

How it is Measured or Detected

TDAR could be examined in vivo and in vitro.

In vivo studies of antigen-specific antibodies are usually performed by measuring serum antibody levels with Enzyme-Linked ImmunoSorbent Assay (ELISA) or with a plaque-forming cell (PFC) assay.

- Rats were repeatedly administered FK506 orally for 4 weeks and immunized with KLH, after which the serum was examined for T-cell–dependent, antigen-specific, IgM and IgG levels using a Sandwich ELISA kit (Ulrich et al. 2004).
- Mice were repeatedly administered calcineurin inhibitors (CNIs) including FK506 and CsA orally for 4 days and immunized with SRBC, after which spleen cells were examined using a PFC assay (Kino et al. 1987).
- Cynomolgus monkeys received 50 mg/kg CsA twice a day via oral gavage (10 h apart) for 23 days and were immunized with SRBC, after which the serum was examined for Anti-SRBC IgM and IgG levels using an ELISA specific for SRBC antigen (Kevin, G. et al. 2014).
- Mice were exposed a single pharyngeal aspiration of DBA, after which supernatants of splenocytes cultured for 24 h in the presence of LPS and assayed using a mouse IgM or IgG matched pairs antibody kit (Bethyl Laboratories, Montgomery, TX) (Donna, C. et al. 2010).

For in vitro studies, total IgM and IgG levels in culture supernatant are often measured after polyclonal T-cell activation rather than measuring antigen stimulation in immune cell cultures.

- T cells and B cells isolated from human peripheral blood mononuclear cells (PBMC) were co-cultured with a CNIs for nine days in the presence of polyclonal–T-cell stimulation, after which supernatants were tested for immunoglobulin IgM and IgG levels using a Sandwich ELISA kit. Treatment with FK506 or CsA reduced the levels of IgM and IgG at the concentrations of 0.3 and 1.0 ng/mL or 50 and 100 ng/mL (Heidt et al, 2009).
- SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) were cultured with anti-CD3/CD28 antibody-stimulated PBMC culture supernatant. After culturing for four days, IgM produced in the culture supernatants was measured using an ELISA kit. FK506 or CsA reduced the levels of IgM at the concentrations of 0.01 to 100 ng/mL or 0.1 to 1000 ng/mL (Sakuma et al. 2001b).
- In order to examine class switching, T cells derived from human PBMCs were cultured with CNIs, and cytokine mRNA levels of Interferon-gamma, IL-2, IL-4, IL-5, IL-10, IL-13, and other B-cell–stimulatory cytokines produced in T cells were measured by quantitative PCR (Dumont et al. 1998).

Regulatory Significance of the AO

TDAR is considered to be the most important endpoint of immunotoxicity, because T cells, B cells, and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR. Thus, changes in any of these immune cell populations can influence TDAR.

Moreover, ICH S8 immunotoxicity testing guideline on pharmaceuticals recommends that TDAR be evaluated whenever the target cells of immunotoxicity are not clear based on pharmacology and findings in standard toxicity studies. For the assessment for pesticides, US EPA OPPTS 870.7800 immunotoxicity testing guideline recommends TDAR using SRBC.

The draft FDA guidance of nonclinical safety evaluation for immunotoxicology recommends TDAR assay.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1508: Inhibition, Calcineurin Activity leads to Interference, nuclear localization of NFAT (https://aopwiki.org/relationships/1508)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	**adjacent**	**Moderate**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculoides	Mus musculoides	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=60742)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

CN is broadly distributed throughout the body, and the structure of CnA and CnB is highly conserved from yeasts to humans (Kincaid. 1993).

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents and other mammalian species (Rao et al. 1997).

FKBP is found in a wide variety of organisms, from prokaryotes to multicellular organisms (Siekierka et al. 1989). Multiple subfamilies of FKBP have been reported, with at least eight types having been found in mammals. FKBP12 is reported to be expressed in B-cells, Langerhans cells, and mast cells as well as in T-cells of humans, mice and other mammalian species.

Cyclophilins have been found in mammals, plants, insects, fungi and bacteria. They are structurally conserved throughout evolution and all have PPIase activity (Wang P et al. 2005).

These facts indicate that CN and immunophilins are conserved among animals and plants although they show multiple physiological functions.

In addition, CNI/immunophilin complex-induced inhibition of CN phosphatase activity resulting in suppression of immune responses is found in humans and mice.

Key Event Relationship Description

The phosphatase activity of calcineurin (CN) is known to be inhibited by CN inhibitors (CNIs) such as FK506 and cyclosporin A (CsA) through the formation of complexes with immunophilins.

Immunophilins of FK506-binding protein (FKBP) and cyclophilin bind with CNIs FK506 and CsA to form complexes, which inhibit CN activity (Barik. 2006).

While FKBP12, FKBP12.6, FKBP13, and FKBP52 are all part of the FK506-binding FKBP family, FKBP12 has a significant involvement in the mechanism of action for FK506-induced immunosuppression (Siekierka et al. 1989, Kang et al. 2008).

FKBP12 is a 12-kDa protein localized in cytoplasm and has been isolated from Jurkat T-cells as a receptor that binds with the FK506 (Bram et al. 1993). FKBP12 has an FK506-binding domain (FKBD) that comprises 108 amino acids, and is expressed in T cells, B cells, Langerhans cells, and mast cells (Siekierka et al. 1990, Panhans-Gross et al. 2001, Hultsch et al. 1991).

Cyclophilin and FKBP both exhibit peptidyl propyl isomerase (PPIase) activity, but the PPIase activity and the inhibition of activity that they indicate are unrelated to CN regulation.

CN is a heterodimer that comprises a catalytic subunit (CnA) and a Ca-binding regulatory subunit (CnB). CnA handles phosphatase activity as well as calmodulin binding, and CnB regulates intracellular calcium and CnA (Klee et al. 1988, Zhang et al. 1996). CnA is a 59kDa protein with a serine-threonine phosphatase domain.

CNI-immunophilin complexes such as FK506/FKBP complexes and cyclophilin/CsA complexes bind directly to CnA in the cell, causing steric hindrance of substrate binding to CN, which in turn inhibits phosphatase activity of CN (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

The nuclear factor of activated T cells (NFAT) is a substrate of CN (Rao et al. 1997).

When T-cell activation takes place, T-cell–receptor-mediated stimulus increases the intracellular concentration of calcium and activates CnB, which subsequently induces CnA phosphatase activation, leading to dephosphorylation of NFAT. In that process, . dephosphorylated SP motifs exposes nuclear localization signal (NLS) and covers nuclear export signal (NES), thereby promoting nuclear localization of NFAT (Matsuda and Koyasu 2000, Zhu and McKeon 1999).

When CN activity is inhibited by the binding of immunophilin complexes, dephosphorylation does not occur in NFAT, thereby interfering with nuclear localization.

Evidence Supporting this KER

Biological Plausibility
The molecular structures and functions of CN and NFAT are evident based on sufficient scientific findings as mentioned above The known mechanisms for inhibition of CN phosphatase activity by FK506, CsA, or other CNIs are initiated by the formation of complexes with their respective immunophilin species. Immunophilins are general classes of proteins that exhibit PPIase activity, but modification of these functions is unrelated to inhibition of CN activity and thus thought to arise in the molecular structure of the complexes (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

As mentioned above, inhibition of CN phosphatase activity interferes with the dephosphorylation of NFAT, which leads to the suppression of its nuclear localization.

Empirical Evidence
Much experimental data is available that supports the inhibition of CN activity induced by CNI/immunophilin complexes, which subsequently suppress nuclear localization of NFAT. In addition, CN phosphatase activity is inhibited by 24 hours treatment with CNI of FK506 and CsA with IC50 values of 0.5 and 5 nM, respectively (Fruman et al.1992).

Also, concentration-dependent reduction of in vitro nuclear localization of NFAT was evident using imaging flowcytometry at the maximum concentration of 1 μM with minimal concentration of 0.1nM (Jurkat human T cell line) or 10nM (T cells from whole blood) after 2 hours treatment of tacrolimus (Maguire et al. 2013). Interference with translocation of NFAT to the nucleus is also detected using gel mobility shift assay to test nuclear extracts and cytoplasmic extracts, in which the examined concentration of FK506 was 10ng/mL (Flanagan et al. 1991).

These findings show that dose responses and temporality of MIE and KE1 seem to be the same.

Uncertainties and Inconsistencies
CN and NFAT are expressed in T cells and other immune cells including B cells, DC, and NKT cells and related to cytokine productions from these immune cells. Also, expression of IL-2 receptors (IL-2R) in DCs are lowered due to the inhibition of CN phosphatase activity by CNI treatment. Of these, reduced production of IL-2 and IL-4 from T cells plays a major role in suppression of TDAR due to lower proliferation, differentiation, and class switching of B cells. There have been no reports of CNI-induced reduction of cytokines other than IL-2 and IL-4 or reduced expression of IL-2R resulting in TDAR suppression.

FKBP12, a specific immunophilin that binds with FK506, is also an accessory molecule that binds to IP3 and Ryanodine receptors, both of which occur in Ca channels located on the membrane of the endoplasmic reticulum and participate in the regulation of intracellular Ca concentration. When binding with FK506, FKBP12 leaves these receptors to increase the influx of Ca2+ from the endoplasmic reticulum to cytoplasm, which should increase CN activity. Treatment with FK506, however, suppresses NFAT nuclear localization. In addition, FKBP12-knock out mice show no changes in immune function, including T-cell function. These facts suggest that the inhibition of CN-NFAT systems induced by FK506 treatment results from direct inhibition of CN phosphatase activity by FK506/FKBP12 complexes and not by affecting Ryanodine and IP3 receptors associated with FKBP12.

Quantitative Understanding of the Linkage

Response-response relationship
MIE:

Dose-response analysis of the effects of FK506 on CN phosphatase activity in mast cell-derived KiSVMC4W cells transfected with human FKBP12 cDNA showed that increased expression of FKBP12 resulted in a greater than ten-fold increase in sensitivity to FK506-mediated inhibition, as indicated by an IC50 value of roughly 2 nM with linear inverse dose-response curve after 1 hour incuvation (Fruman et al.1995). Another phosphatase assay showed that FK506 inhibition of CN activity was concentration-dependent reverse sigmoidal and that IC50 values for CN inhibition were approximately 0.5 nM for FK 506 and 5 nM for CsA after 1 hour culture (Fruman et al.1992).

KE1:

Dose-dependent interference with nuclear translocation of NFAT1 was observed with increasing CNI concentrations from 0.1 nM (Jurkat human T cells) up to 1 μM (1000 nM) using imaging flowcytometer. Higher concentrations induced cellular toxicity and resulted in cell death. Dose-dependent interference of nuclear NFAT1 translocation per CN inhibition was also observed in CD4+ T cells from healthy donors, again at maximal concentrations of 1 μM with minimum concentration of 10nM (Maguire et al. 2013).

There have been no literature available to compare directly the dose response of inhibition of CN phosphatase activity with that of nuclear translocation of NFAT; however, the concentration ranges of CNIs for inhibition of CN phosphatase activity and nuclear translocation of NFAT seem to be the same range.

Time-scale
Inhibition of CN phosphatase activity was examined after 1 hour culture of T cells (Fruman et al.1995, Fruman et al.1992), and inhibition of nuclear translocation of NFAT was measured by imaging flowcytometry after 2 hour culture of T cells with CNI (Maguire et al. 2013).

Known modulating factors
At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER
At present, no evidence is found.

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Relationship: 1017: Interference, nuclear localization of NFAT leads to Reduction, NFAT/AP-1 complex formation (https://aopwiki.org/relationships/1017)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	**adjacent**	**High**	**High**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents, and other mammalian species (Rao et al. 1997).

CN-NFAT system functionality is common among mammalian species, including humans and rodents. It is also possible that FK506-induced interference with NFAT/AP-1 complex formation at the promoter site of the IL-2 gene is common among mammalian T cells, including those of humans and rodents (Flanagan et al. 1991).

Key Event Relationship Description

Activated (dephosphorylated) nuclear factor of activated T cells (NFAT) is translocated into the nucleus through the molecular changes of exposing nuclear localization signal (NLS) and concomitant masking of nuclear export signal (NES) due to dephosphorylation of the SP motifs of NFAT. (Matsuda and Koyasu 2000, Zhu and McKeon 1999).

Nuclear localization of NFAT results in the NFAT binding with AP 1 at the IL-2 promoter region, (Schreiber and Crabtree 1992; Jain et al. 1992) and induces transcription of IL-2 (Jain et al. 1993). In addition to IL-2, NFAT localized in the nucleus of T cells also binds to the promoter region of the other classes of cytokines including IL-4 and IL-13.

Once CN phosphatase activity is inhibited, dephosphorylation of NFAT and subsequent nuclear localization of NFAT decreases, which results in a decrease of NFAT/AP-1 complex formation at the cytokine promoter sites (Rao et al. 1997).

Evidence Supporting this KER

Biological Plausibility
As has been mentioned, NFAT has NLS and NES among and adjacent to the N-terminal region rich in SP motifs, and once the SP region is dephosphorylated, the NLS is exposed and the NES is covered, which leads to translocation of NFAT into the nucleus (Matsuda and Koyasu 2000).

It is well known from the experiments using CN inhibitors (CNIs) that interference with the nuclear localization of NFAT in T cells leads to a reduction in the formation of NFAT/AP-1 complexes, thereby suppressing transcription of IL-2, IL-4, and a number of other cytokines (Maguire et al. 2013, Jain et al. 1992, Jain et al. 1993).

In contrast to T cells, B-cell receptor-mediated increases in intracellular concentration of calcium in B cells leads to NFAT nuclear localization, thereby producing some classes of cytokines in the same manner as T-cells (Bhattacharyya et al.2011). However, there has been no report of any evidence that CNI acts directly on B cells to effect antibody production.

Expression of IL-2 receptors in dendritic cells and NKT cells is also reported to be regulated by this CN-NFAT system (Panhans-Gross A et al. 2001; Kim et al. 2010), but there is no report showing that CNIs suppress TDAR through the changes in IL-2R expression in these cells.

Empirical Evidence
The relationship of the interference of nuclear localization of NFAT leading to reduced NFAT/AP-1 complex formation bound at the promoter sites of cytokine genes in the presence of CNIs is well known as mentioned above.

Imaging flowcytometry revealed that concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the maximum concentration of 1 μM with minimal concentration of 0.1nM (Jurkat human T cell line) or 10nM ($CD4^+$T cells from whole blood) after 2 hours treatment of tacrolimus (Maguire et al. 2013).

The experiment of gel mobility shift assay using Ar-5 human T cells stimulated with cross-linked anti-CD3 antibody showed that NFAT/AP-1 (cFos and Jun) complexes were found only in the nuclear extract with preexisting NFAT in the cytoplasm after T cell stimulation and that the NFAT/AP-1 complexes in the nucleus decreased after 2 hours treatment with CsA at 1μM (Jain et al. 1992). Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991) NFAT/AP-1 complex formation was also reported to be inhibited by CNI (Rao et al. 1997).

Quantitative understanding of NFAT/AP-1 complex formation in the nucleus is insufficient although nuclear NFAT/AP-1 complex formation was shown to be inhibited with FK506 at the concentration within the concentration range of FK506 for the inhibition of nuclear translocation of NFAT.

Uncertainties and Inconsistencies
Nothing especially

Quantitative Understanding of the Linkage

Response-response relationship
The relationship of the interference of nuclear localization of NFAT leading to reduced NFAT/AP-1 complex formation bound at the promoter sites of cytokine genes in the presence of CNIs is well known as mentioned above.

KE1:

Dose-dependent interference with nuclear translocation of NFAT1 was observed with increasing FK506 concentrations from 0.01nM (Jarkat T cells) up to 1 μM (1000 nM). Higher concentrations induced cellular toxicity and resulted in cell death. Dose-dependent interference of nuclear NFAT1 translocation per CN inhibition was also observed in CD4+ T cells from healthy donors, again from 10nM to maximal concentrations of 1 μM (Maguire et al. 2013). Both parameters were measured after 2 hour culture of T cells with FK506.

KE2:

Reduction in generation of NFAT/AP-1 complexes can be detected using a gel shift assay (Rao et al. 1997, Jain et al. 1992, Jain et al. 1993).

Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991). As mentioned above, the gel mobility shift assay also showed that NFAT/AP-1 complexes were formed only in the nucleus after T cell activation with unchanged preexisting NFAT in the cytoplasm and that treatment of T cells with 1μM FK506 led to decease the levels of NFAT/AP-1 complex (Jain et al. 1992).

These findings suggest that nuclear translocation of NFAT after T cell stimulation is strongly related to the complex formation with AP-1 in the nucleus, and FK506 was shown to inhibit NFAT/AP-1 complex formation in the nucleus at the concentrations within the concentration range of FK506 for suppressing nuclear translocation of NFAT (Maguire et al. 2013).

Time-scale
Nuclear translocation of NFAT was shown to be inhibited in vitro using imaging flowcytometry after 2 hours culture of T cells with FK506 (Maguire et al. 2013), and gel mobility shift assay revealed the inhibition of nuclear translocation of NFAT and following complex formation with AP-1 within the nucleus after 2 hours culture of T cells with FK506 (Jain et al. 1992, Flanagan et al. 1991).

Known modulating factors
At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER
At present, no evidence is found.

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Relationship: 1509: Reduction, NFAT/AP-1 complex formation leads to Suppression, IL-2 and IL-4 production (https://aopwiki.org/relationships/1509)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	**adjacent**	**High**	**High**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

In purified T cell from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1, IL-2 production and CD25 (IL-2R) up-regulation (Yoshida et al. 2015).

In splenic lymphocytes and/or CD4+ T cells, ursolic acid suppressed products of NF-kB, NFAT and AP-1, and inhibits secretion of IL-2 and IL-4, mRNA level of IL-2 and CD25 expression (Checker et al. 2012).

NFATp- and NFAT4-deficient mice indicate decreased production of IL-2 (Ranger et al. 1998).

NFAT/AP-1 complex formation in the nucleus was shown using murine and human T cells lines (Jain J et al. 1992). In addition to data on suppression of cytokine production by CNI in rodents, FK506 is reported to inhibit expression of both IL-2 and mRNA in human anti-CD3/PMA-activated cells (Dumont et al. 1998).

Key Event Relationship Description

Localized nuclear factor of activated T cells (NFAT) in the nucleus of T cells binds to form complexes with activator protein-1 (AP-1) at the Interleukin (IL)-2 promoter region (Schreiber and Crabtree 1992; Jain et al. 1992), which induces transcription of IL-2 (Jain et al. 1993). In addition to IL-2, NFAT localized in the nucleus of T cells also binds to the promoter region of the other classes of cytokines including IL-4 and IL-13.

For IL-2, NFAT proteins are necessary for IL-2 gene expression and cooperation of NFAT with AP-1 is required for IL-2 gene transcription. For IL-4, At least five different NFAT sites have been described in the IL-4 promoter with at least three of them being composite sites binding NFAT and AP-1 (Macián et al. 2001).

Decreased formation of NFAT/AP-1 complex at the promoter region of IL-2 genes in the nucleus of T cells following lowed nuclear localization of NFAT by calcineurin inhibitor (CNI) treatment reduces the transcription of IL-2 (Dumont et al. 1998). Production in T cells of IL-4 and other classes of cytokines is also suppressed in the same manner as IL-2 (Dumont et al. 1998).

Evidence Supporting this KER

Biological Plausibility

T-5224, a selective c-Fos/AP-1 inhibitor, inhibits the DNA-binding activity of AP-1 in primary murine T cells. T-5224 also inhibits CD25 (one of IL-2 receptors) up-regulation, IL-2 production, and c-Fos DNA-binding activity in mice (Yoshida et al. 2015).

Dexamethasone represses the IL-2 mRNA induction. glucocorticoid-induced leucine zipper (GILZ) is one of the most prominent glucocorticoid-induced genes, and inhibited the induction of the NFAT reporter and interferes with the AP-1 component of the NFAT/AP-1 complex. GILZ also inhibits the IL-2 promoter (Mittelstadt et al. 2001).

Ursolic acid suppressed activation of three immunoregulatory transcription factors NF-kB, NFAT and AP-1. Treatment of lymphocytes and CD4+ T cells with ursolic acid inhibited secretion of IL-2 and IL-4 cytokines. Treatment of CD4+ T cells with ursolic acid suppressed mRNA level of IL-2. Treatment of lymphocytes with ursolic acid inhibited the upregulation of CD25 expression on T cells (Checker et al. 2012).

NFATp- and NFAT4-deficient mice indicate decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998).

It is generally accepted that NFAT, translocated to the nucleus after T-cell stimulation, binds with AP-1 to the promoter regions of the cytokine genes to mount transcription, which follows production of these T-cell–derived cytokines. Of these cytokines, IL-2 and IL-4 promote proliferation, maturation, and class-switching of B cells to enhance TDAR.

There is also sufficient evidence to support the hypothesis that CNI-induced decreases in T-cell–derived cytokine production is mediated through suppressed nuclear localization of NFAT, with a resultant decrease in the amount of NFAT/AP-1 complex binding to the promoter regions of T-cell-derived cytokines.

When stimulated with ovalbumin, calcineurin A (CnA)-knockout (KO) mice produce less Interferon (IFN)- γ, IL-2, and IL-4 than wild-type mice. However, primary antibody response in CnA-KO mice is normal in response to trinitrophenol-ovalbumin (Zhang et al. 1996).

The following phenotypes are observed in NFAT-KO mice: moderate hyperproliferation with splenomegaly; moderately enhanced B- and T-cell responses, with bias towards Th2- cell responses; decreased IFN-γ production in response to TCR ligation; reduced proliferative responses by T cells; impaired repopulation of the thymus and lymphoid organs; impaired Th2-cell responses and IL-4 production; grossly impaired T-cell effector functions, with profound defects in cytokine production and cytolytic activity; B-cell hyperactivity; impaired development of CD4 and CD8 single-positive cells, with increased apoptosis of double-positive thymocytes; and mild hyperactivation of peripheral T cells (Macian, 2005).

Therefore, the study of NFAT-KO mice shows that NFAT is involved in a wide range of immune responses, and some of these phenomenon are known to be regulated by calcineurin (CN). Suppression of T-cell-derived cytokines is noted both in CnA-KO and NFAT-KO mice, which indicates that the production of T-cell derived cytokines such as IL-2 and IL-4 is regulated by the CN-NFAT system.

FK506-FKBP12 complex decreased CN phosphatase activity, which leads to inhibit translocation of NFAT to the nucleus. Because NF-ATp is an essential transcription factor regulating the IL-2 gene, FK506 ultimately blocks the T-cell response by inhibiting IL-2 transcription (Panhans-Gross A et al. 2001). FK506 inhibited IL-2 mRNA expression in anti-CD3/phorbol 12-myristate-13-acetate (PMA)-activated cells (Dumont et al. 1998).

These facts indicate that although NFAT is widely involved in the function of T cells, the effect of CNIs is to suppress production of some classes of T-cell–derived cytokines through reducing the formation of NFAT/AP-1 complexes induced by inhibition of CN phosphatase activity.

Empirical Evidence
Empirical support of Reduction, NFAT/AP-1 complex formation leading to Suppression, IL-2 and IL-4 production is strong.

Rationale

- In purified T cell from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 and CD25 (one of IL-2 receptors) up-regulation at 80 μg/mL, and IL-2 production in a dose-dependent manner from 40 to 80 μg/mL (Yoshida et al. 2015).
- In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 μM for 4 h. Secretion of IL-2 and IL-4 was inhibited in lymphocytes stimulated with concanavalin A for 24 h at concentrations of 0.5, 1 and 5 μM of ursolic acid, and lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h at concentration of 5 μM of ursolic acid. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 μM for 4 h. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 μM for 4 h (Checker et al. 2012).
- In NFATp- and NFAT4-deficient mice, cultured splenocytes bound anti-CD3 for 48 h indicates decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998).

It is well established that inhibition of NFAT/AP-1 complex formation at the promoter sites reduces the production of T-cell–derived cytokines including IL-2 and IL-4, which are mainly involved in T-cell–dependent antibody response.

- NFAT/AP-1 complex formation is inhibited by CNI shown by gel shit mobility assay using human T cell line or CD4+ T cells from heathy donners after 2 hours treatment with cyclosporin A (CsA) at 1μM.. Preceding NFAT nuclear localization after T cell activation is suppressed with FK506 at the dose range of 0.01nM (Jarkat T cells) or 10nM (CD4+ T cells) to 1μM (Maguire et al. 2013), and NFAT nuclear localization and NFAT/AP-1 complex formation is shown to be strongly related (Jain et al. 1992, Jain et al. 1993).
- In CD3/PMA-activated human T cells, FK506 suppressed production of IL-2, IL-4, and IFN-γ at the concentrations of 1.2 to 12.5 nM after 22 to 24 hours culture as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA in a dose-dependent (10 nM) manner after 3 day culture (Dumont et al. 1998).
- Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC25 and IC50 for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC25 and IC50 for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018).
- In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010).
- Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).
- In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) reduced IL-2 release in response to keyhole limpet hemocyanine (Alessandro, B. et al. 2003).

Reduced nuclear translocation of NFAT followed by NFAT/AP-1 complex formation and suppression of IL-2/IL-4 productions are shown to occur under similar dose ranges and treatment duration.

Uncertainties and Inconsistencies
CNIs are reported to suppress IL-17 release from Th17 cells and development of Th17 cells from naïve T cells (Tsuda et al, 2012). On the other hand, Yadav reported that Th17 cells increased and Treg cells decreased in number and that the levels of RORC mRNA increased and those of FOXP3 decreased in renal transplanted patients with chronic calcineurin inhibitor toxicity (Yadav, 2015). From these findings, CNIs suppress the functions of Th17 and Treg cells which enhance Th17 cells to develop chronic CNI toxicity.

FK506 suppresses expression of IL-2 receptor (IL-2R: CD25) and costimulatory molecules CD80 (B7.1)/CD40 in Langerhans cells (Panhans-Gross A et al. 2001).

In human NK cells, FK506 suppresses IL-2 responsive proliferation and cytokine production as well as lowers cytotoxicity directed toward K562 tumor cells (Kim et al. 2010). FK506 suppresses IL-2 production of NKT cell line DN32.D3 induced by stimulus from PMA/calcium -ionophore (van Dieren et al. 2010).

The relationship between these FK506-induced mechanisms and NFAT and contribution of those to TDAR are unclear.

In addition to NFAT/AP-1 complexes, NFAT forms complexes at the site of IL-3 and IL-4 enhancers with avian musculoaponeurotic fibrosarcoma oncogene homolog, early growth response 1, early growth response 4, interferon-regulatory factor 4, octamer-binding transcription factor, and other transcriptional partners to induce transcription of a variety of cytokines (Macian 2005). The production of cytokine induced by these transcriptional partners also suppressed by CNI; however, contribution of these additional transcription factors to TDAR is also unclear.

Quantitative Understanding of the Linkage

Response-response relationship

In purified T cells from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 at 80 μg/mL. On the other hand, T-5224 inhibits IL-2 production in a dose-dependent manner from 40, 60 and 80 μg/mL after 48 hours culture. T-5224 also inhibits CD25 (IL-2R) up-regulation at 80 μg/mL (Yoshida et al. 2015).

In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 μM. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibits secretion of IL-2 and IL-4 at 0.5, 1 and 5 μM. In lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid also inhibits secretion of IL-2 and IL-4 at 5 μM. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 μM. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 μM (Checker et al. 2012).

These findings showed that T-5244 and ursolic acid treated for 24 hours inhibit NFAT/AP-1 complex formation at a single concentration each and that these compounds suppress IL-2 and IL-4 production with dose dependent manner including the doses for inhibition of NFAT/AP-1 complex formation.

FK506 suppressed proliferation in human T cells induced by anti-CD3 mAb in the presence of adherent autologous peripheral blood mononuclear cells (mean IC50 = 0.06 nM). FK506 suppressed, in a dose-dependent (1.2 to 12.5 nM) manner after 22-24 hours culture, production of IL-2, IL-4, and IFN-γ by human T cells stimulated with anti-CD3 mAb in the presence of PMA, as well as inhibited, also in a dose-dependent (10 nM) manner, expression of IL-2, IL-4, and IFN-γ mRNA in anti-CD3/PMA- activated cells (Dumont et al. 1998). On the other hand, the quantitative data for the decreased formation of NFAT/AP-1 complexes by CNI is insufficient, although the formation was suppressed by FK506 at the concentration within the range needed for suppressed production of IL2/IL-4 by FK506 after 2 hours culture.

Time-scale

Inhibition of NFAT/AP-1 complex is detected by gel mobility shit assay after 2 hours culture with CNI; however, suppression of IL2/IL-4 could be measured after 22-48 hours in vitro culture.

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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Relationship: 1510: Suppression, IL-2 and IL-4 production leads to Impairment, T-cell dependent antibody response (https://aopwiki.org/relationships/1510)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	**adjacent**	**High**	**High**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
cynomolgus monkey	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

In cynomolgus monkeys, the effects of CsA on production of IL-2 and IL-4, and antigen-specific IgM and IgG in TDAR were demonstrated (Gaida K. 2015).

Suppressed IgE and antigen specific IgG1 productions by the blocking of IL-4 receptor were reported in mice using dupilumab (anti-IL-4/13R antibody) (Sanofi K.K. 2018).

Suppressed antigen specific IgE production by the inhibition of IL-4 production was reported in mice using suplatast tosilate (Taiho Pharmaceutical 2013).

Suppressed antigen specific IgE and IL-4 productions by the inhibition of IL-4 production were reported in human cell culture using suplatast tosilate(Taiho Pharmaceutical 2013).

The effects of FK506 on serum concentration of anti-KLH antibodies IgM and IgG have been demonstrated in rats treated with FK506 for over four weeks and immunized with KLH (Ulrich et al. 2004). The effects of FK506 and CsA on antigen-specific plaque-forming splenocytes have been demonstrated in mice treated with FK506 or CsA for 4 days and immunized with SRBC (Kino et al. 1987b).

The effects of FK506 and CsA on the levels of IgM and IgG in the culture supernatant have been demonstrated in human cells (Heidt et al, 2009, Sakuma et al, 2001).

The effects of FK506 and CsA on production of IL-2 and IL-4 have been demonstrated using mice and human cells (Kino et al. 1987a, Dumont et al. 1998).

These facts suggest that there are no species differences between humans, monkeys and rodents in inhibitions of IL-2 and IL-4 production and TDAR induction.

Key Event Relationship Description

Interleukin (IL)-2 and IL-4 are produced and secreted by helper T cells and play important roles in the development of T-cell dependent antibody response (TDAR), both of which induces/enhances T cell dependent antibody production. IL-4 affects maturation and class switching of B cells as well as proliferation, IL-2 promotes differentiation of B cells through IL-2 receptors and stimulates the activated T cell into T cell called Th2 cell. Therefore, suppressed production of IL-2 and IL-4 impairs T cell dependent antibody production (Alberts et al. 2008).

T cells, B cells, and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR. Thus, changes in any of these immune cell populations can influence TDAR

T cell-derived cytokines play important roles in the development of TDAR. Among them, IL-2 promotes proliferation of B cells, and IL-4 affects maturation and class switching of B cells as well as proliferation, both of which induces/enhances T cell dependent antibody production.

Thus, suppressing the production of IL-2, IL-4, and other cytokines in T cells reduces stimulation of B cells including proliferation, activation, and class switching, and leading to impairment of TDAR. Therefore, suppressing the production of these B-cell-related cytokines appears to be the main factor in impairment of TDAR by inhibitors of T-cell–dependent-antibody production.

Evidence Supporting this KER

Biological Plausibility
Cyclosporin A (CsA) is known one of the calcineurin inhibitiors. CsA-treatment is reported to suppresses the productions of IL-2 and IL-4 and result in the reduction of the productions of antigen-specific IgM and IgG in cynomolgus monkeys (Gaida K. 2015).

It is established that IL-2 stimulates B cells to proliferate through the surface IL-2 receptors and that IL-4 stimulates B cells to proliferate, to induce class switch, and to differentiate into plasma and memory cells.

Dupilumab is known as anti-IL-4/13 receptor (IL-4/13R) antibody. Dupilumab (Dupixent) reduces productions of immunoglobulin (Ig) E and antigen specific IgG1 in mice (Sanofi K.K. 2018). It suggests that the blocking of IL-4 signaling by anti-IL-4/13R antibody results in the decrease in T cell dependent antibody production.

Th2 cell produces cytokines including IL-4. Suplatast tosilate (IPD) is known as an inhibitor of the production of IL-4 and IL-5 from Th2 cells and reduces the production of antigen specific IgE in human cell culture and mice (Taiho Pharmaceutical 2013). These findings suggests that the reduction of IL-4 production by the inhibitor of Th2 cell cytokines results in reduced production of IgE and/or IgG1 through inhibitions of maturation, proliferation and class switching of B cells.

IL-2 binds to IL-2 receptor (IL-2R) and acts on T cell. CD25 is one of IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016). They suggest that the blocking of IL-2 signaling by anti-IL-2R antibody results in decreased rejection through the inhibition of the activation of antigen specific T cell with reduced antibody production.

FK506 and CsA suppress mRNA expression levels of cytokines in T cells including IL-2 and IL-4 that stimulate proliferation of B cells as well as B cell activation and class switching (Heidt et al, 2010).

Several in vivo studies in rodents showed decreased TDAR by the treatment of FK506 (Kino et al. 1987b, Ulrich et al. 2004). In in vitro tests examining antibody production in blood samples obtained from blood-bank donors, peripheral blood mononuclear cells (PBMC) treated with FK506 and CsA suppressed the production of IgM and IgG antibodies to T-cell dependent antigens (Heidt et al, 2009).

T cells, B cells, and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR. Thus, changes in any of these immune cell populations can influence TDAR.

However, as for the suppression of humoral immunity induced by the inhibition of calcineurin (CN) phosphatase activity, calcineurin inhibitors (CNIs) do not affect B cells directly but rather indirectly through T cells. That is, FK506 and CsA are capable of inhibiting immunoglobulin production when B cells are cultured with non-pre-activated T cells, but FK506 and CsA fail to inhibit immunoglobulin levels when pre-activated T cells are used to stimulate B cells. Hence, the inhibition of B cell response by FK506 and CsA appears due solely to inhibition of T helper cells (Heidt et al, 2010).

Therefore, it is concluded that decreased amounts of IL-2 and IL-4 secreted from helper T cells is the main factor for suppression of TDAR induced by CN phosphatase inhibition.

Empirical Evidence
Empirical support of the suppression, IL-2 and IL-4 production leads to impairment, T-cell dependent antibody response is strong.

Rationale

- Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015).
- In the allergen-induced pneumonia model in mice, dupilumab (anti-IL-4/13R antibody) reduced productions of IgE and antigen specific IgG1 at 25 mg/kg of twice weekly subcutaneous administration for 4weeks (Sanofi K.K. 2018).
- In mice immunized with dinitrophenyl antigen by i.p. injection, suplatast tosilate (an inhibitor of the production of cytokines on Th2 cell) reduced productions of antigen specific IgE at 10, 20, 50 and 100 mg/kg of oral administration for 5 days (Taiho Pharmaceutical 2013). In human cell culture immunized with Japanese cedar antigen, suplatast tosilate reduced productions of antigen specific IgE at the concentration of 10 μg/mL for 10 days (Taiho Pharmaceutical 2013).
- In the clinical study of renal transplantation, basiliximab decreased incidence of acute rejection at 20 mg/kg (Novartis Pharma 2016). In human T cell culture immunized with PPD, basiliximab reduced activation of antigen specific T cell at the concentration of 300 ng/mL (Novartis Pharma 2016).

- In CD3/phorbol 12-myristate-13-acetate-activated human T cells, FK506 suppressed production of IL-2, IL-4 and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM as well as inhibited expression of IL-2, IL-4 and IFN-γ mRNA at the concentrations of 10 nM. (Dumont et al. 1998).
- FK506 or CsA suppressed production of IL-2 in mouse mixed lymphocyte reaction (MLR) at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA as well as in human MLR at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA (Kino et al. 1987a).
- After 9-day culture of B cells and non-pre-activated T cell stimulationwith FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41 and 83nM) of CsA (Heidt et al, 2009).
- After 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at the concentrations of 0.01 to 100 ng/mL (0.01 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.08 to 832 nM) of CsA (Sakuma et al, 2001).
- Rats were treated with FK506 for over four weeks and immunized with keyhole limpet hemocyanine (KLH), after which serum concentration of anti-KLH IgM and IgG reduced at the dose levels of 3 mg/kg/day (Ulrich et al. 2004).
- Mice were treated with FK506 or CsA for 4 days, and immunized with sheep red blood cells (SRBC), after which antigen-specific plaque-forming splenocytes reduced at the dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b).
- 1,2:5,6-dibenzanthracene single adoministration suppressed production of IL-2 and total IgG antibody in mice at the dose levels of 3 and 30 mg/kg(Donna, C. et al. 2010).
- In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) for 21 days reduced IL-2 release in response to KLH and decrease in anti-KLH IgG(Alessandro, B. et al. 2003).

In vitro suppression of T-cell–derived cytokines and T-cell-dependent antibody production or antibody production after polyclonal T-cell stimulation showed similar dose responses to CNIs. Time gaps were found, however, between these two KEs, which showed earlier onset of cytokine production and delayed onset of antibody production.

Uncertainties and Inconsistencies
IL-2 affects multiple populations of immune cells expressing IL-2 receptors, while IL-4 mainly acts on B cells. Therefore, reduced production of both IL-2 and IL-4 might certainly induce suppression of TDAR; however, there remains some possibility of additional suppression of other immune functions.

Quantitative Understanding of the Linkage

Response-response relationship
Cynomolgus monkeys treated wth CsA at 50 mg/kg BID showed suppression of IL-2 and IL-4 production and inhibition of SRBC-specific IgM and IgG in TDAR (Gaida K. 2015).

In the blocking of IL-4 receptor in mice by dupilumab (anti-IL-4/13R antibody) at 25 mg/kg of twice weekly subcutaneous administration for 4weeks, IgE production was suppressed to about 1/100 and antigen specific IgG1 production was suppressed to about 1/200 (Sanofi K.K. 2018).

In the inhibition of IL-4 production in mice by suplatast tosilate at 10, 20, 50 and 100 mg/kg of oral administration for 5 days, antigen specific IgE production was suppressed from about 1/10 to 1/100 (Taiho Pharmaceutical 2013). In human T cell culture by suplatast tosilate at the concentration of 10 μg/mL, antigen specific IgE production after 10 days was suppressed from 56 to 72% and IL-4 production after 3 days was suppressed from 58 to 76% (Taiho Pharmaceutical 2013).

As for IL-2 and antibody production, in vitro T-cell-induced polyclonal B cell activation to produce antibody was inhibited with anti-IL-2 and anti-IL-2R antibodies. That is, murine small resting B cells, cultured with irradiated hapten-specific TH1 clone, were induced to enter cell cycle at 2 days and to secret antibody at 5 days. An anti-IL-2 and anti-IL-2R antibodies completely inhibited this T-cell dependent antibody production (Owens T, 1991).

In the human T-B cell co-culture stimulated with anti-CD3 monoclonal antibody, CNIs of FK506 and CsA lowered the m-RNA levels of T-cell cytokines at 8h post-stimulation including IL-2 and IL-4 at 1.0ng/mL (1.24nM) FK506 or 100ng/mL (90.7nM) CsA and inhibited IgM and IgG productions after 9 days at 0.3 and 1.0ng/mL FK506 and 50 and 100ng/mL CsA (Heidt S. 2010).

Time-scale
In CsA-treatment for 24 days at 50 mg/kg BID, cynomolgus monkeys showed suppression of IL-2 and IL-4 production and inhibition of SRBC-specific IgM and IgG in TDAR (Gaida K. 2015).

In human T cell culture, suplatast tosilate inhibits IL-4 production after 3 days and antigen specific IgE production after 10 days (Taiho Pharmaceutical 2013).

In the human T-B cell co-culture, CNIs of FK506 and CsA lowered the m-RNA levels of IL-2 and IL-4 at 8h post-stimulation and inhibited IgM and IgG productions after 9 days (Heidt S. 2010).

Known modulating factors
At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER
At present, no evidence is found.

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AOP ID and Title:

SNAPSHOT

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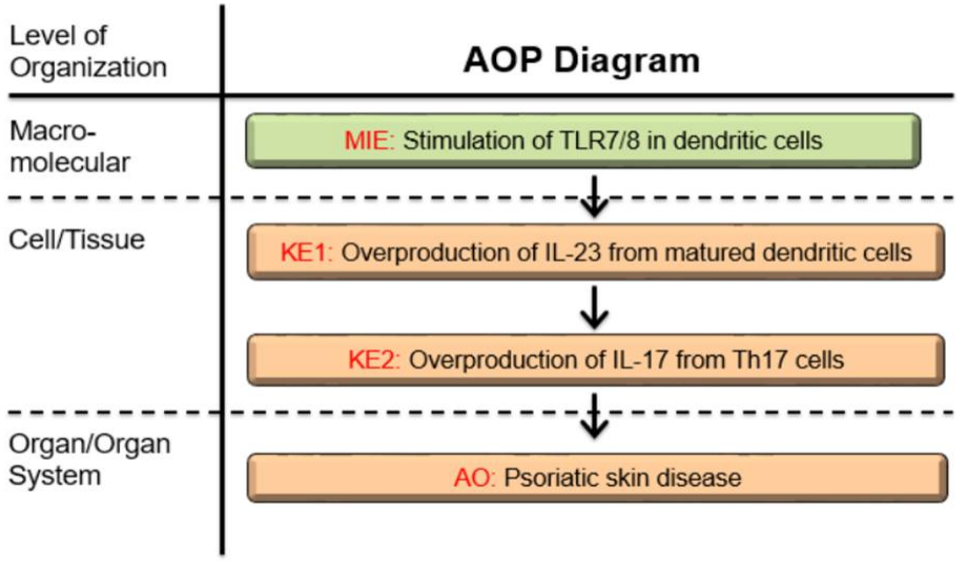
AOP 313: Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease

Short Title: Skin disease by stimulation of TLR7/8

Graphical Representation



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Abstract

Toll-like receptor (TLR) 7 and TLR8 are pattern recognition receptors that are known to activate antiviral reaction of immune system, hyperactivation of which can lead to psoriatic skin disease when hyperactivation of them occurred. The relationship between TLR7/8 and immune functions is well understood, and antiviral compound that work by stimulating TLR7/8 have been developed. TLR7/8 agonists such as imidazoquinolin compounds stimulate these TLRs through the formation of homodimer. This signal activates the IL-23/IL-17 axis, which leads to psoriasis and other related skin diseases.

Activation of the IL-23 / IL-17 axis and causes abnormal proliferation and inflammation of the epidermis, which is a pathological condition of psoriasis. This AOP shows an association between TLR7 / 8 stimulation and psoriatic skin disease.

TLR7-mediated signaling in plasmacytoid dendric cells (pDC) is mediated in a MyD88-dependent fashion, which initiates an IRF7, IRAK1, TRAF6, TRAF3, and IKKα-mediated response, secreting vast amounts of IFN type 1. Similarly, upon engagement of ligands in endosomes, TLR8 initiate the MyD88-dependent pathway culminating in synthesis and release of proinflammatory mediators, such as TNF-α via NF-κB activation. IFN-α and TNF-α cooperatively mature myeloid dendritic cells. TLR7/8 agonist stimulates a specific population of inflammatory dermal dendritic cells referred as TNF and inducible nitric oxide synthase–expressing DCs (Tip-DCs) to produce IL-23 after maturation by enhanced transcriptional activity.

IL-23R is mainly expressed in Th17 cells. In chronic psoriasis, the cytokines IL-12 and IL-23 produced by resident DC are the main causes. Not only does the expression of IL-23 increases in the skin tissue of the lesion, Th17 cells also increase.

Mature Th17 cells are activated by IL-23 stimulation. Signaling through IL-23 produces cytokines IL-17 and IL-22 that mediate the psoriasis response and promote neutrophil migration into the epidermis, epidermal cell proliferation, and similar responses, which lead to the development of a psoriasis rash. In mice, psoriasis-like hyperplasia is induced by the application of IL-23 but does not occur in IL-17A and IL-22 KO mice, so IL-17A and IL-22 play an important role downstream of IL-23.

IL-17 receptor form heterodimers, and IL-17RA / IL-17RC appears in a variety of cells, including fibroblasts and epidermal cells. IL-17RE / IL-17RA expressed in epidermal cells and IL-17C binding are also important in the pathology of psoriasis. Immunohistochemically, IL-17A is expressed only in cells of the dermal papilla layer, while IL-17C is widely expressed in cells such as hyperproliferative overexpressed keratinocytes, leukocytes, and vascular endothelial cells. IL-17C produces keratinocytes by bacterial stimulation and further stimulates keratinocytes to induce the production of various cytokines and chemokines. Keratinocytes are known to be self-activated by IL-17C.

IL-17 and IL-22 secreted from Th17 act on keratinocytes, causing abnormalities in keratinocytes through the secretion of inflammatory cytokines, chemokines, growth factors, and antimicrobial peptides, and thereby exacerbating the skin symptoms of psoriasis.

The creation of this AOP began with an examination of important event relationships brought about by TLR7 / 8 activity due to environmental or genetic factors and resulting in abnormal differentiation of keratinocytes, which leads to thickening of the epidermis and its resultant autoimmune skin disease, psoriasis

Background

Psoriasis is an chronic autoimmune disease characterized by chronic epithelial inflammatory disease induced by environmental factors such as infection, stress, smoking or alcohol consumption as well as by genetic factors. The onset of psoriasis has been reported to be triggered by drugs and chemical substances use, including beta-blockers, chloroquine, lithium, ACE inhibitors, indomethacin, terbinafine, and interferon alpha. Diagnosis is based on the type and distribution of the lesions.

Psoriasis occurs when abnormal differentiation (keratosis) of keratinocytes leads to thickening of the epidermis. Patients often exhibit an erythema with a clear border and epidermal hyperplasia, stratum corneum hyperplasia, heterocytosis in the stratum corneum, mixed skin moist cells of neutrophilic granulocytes and T cells in the epidermis. Dendritic cells (DC) and macrophages are associated with silver-white plaque. Neutrophilic effusion (Munro microabscesses) are observed in the epidermis, and CD8+ T cells (Tc17) increase the expression of angiogenesis related genes.

The main therapeutic agents are mild topical treatments such as emollients, salicylic acid, coal tar preparations, anthralin, corticosteroids, vitamin D3 derivatives, retinoids, calcineurin inhibitors or tazarotene. UV therapy is also used for moderate or severe psoriasis. Widespread psoriasis is treated with systemic therapies such as immunomodulators methotrexate, cyclosporin, retinoids and other immunosuppressants used alone or in combination.

Although there are stressors that are well known to induce psoriasis-like skin inflammation in mice, this AOP is based primarily on an understanding of stimulation caused by imiquimod, resiquimod or LL37-selfRNA complexes, for which a significant body of scientific literature has been published.

As a test model for psoriasis, an Autoimmune skin disease, mouse tests that induce skin inflammation like psoriasis are frequently conducted using the imidazoquinoline derivative imiquimod. This AOP is primarily based on an understanding of stimuli caused by imiquimod, resiquimod, or LL37-selfRNA complexes.

Imiquimod is derived from imidazoquinoline and is often used to create mouse models. It is our hope that this AOP will contribute to greater knowledge about the development of psoriatic skin diseases that start from stimulation of TLR as well as the development of new treatment targets for psoriasis.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	1706	Stimulation, TLR7/8 in dendritic cells (https://aopwiki.org/events/1706)	Stimulation of TLR7/8
2	KE	1707	Overproduction of IL-23, matured dendritic cells (https://aopwiki.org/events/1707)	Overproduction of IL-23
3	KE	1708	Overproduction of IL-17 from Th17 cells (https://aopwiki.org/events/1708)	Overproduction of IL-17
4	AO	1709	Psoriatic skin disease (https://aopwiki.org/events/1709)	Skin disease

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Stimulation, TLR7/8 in dendritic cells (https://aopwiki.org/relationships/2017)	adjacent	Overproduction of IL-23, matured dendritic cells	High	High
Overproduction of IL-23, matured dendritic cells (https://aopwiki.org/relationships/2018)	adjacent	Overproduction of IL-17 from Th17 cells	High	High
Overproduction of IL-17 from Th17 cells (https://aopwiki.org/relationships/2019)	adjacent	Psoriatic skin disease	High	High

Stressors

Name	Evidence
Imiquimod	High
Resiquimod	High

Overall Assessment of the AOP

TLR7/8 is stimulated when imidazoquinolin compounds or stimilar agonists from homodimers TLR7-mediated signaling in plasmacytoid dendritic cells (pDC) is mediated in a MyD88-dependent fashion, which initiates an IRF7, IRAK1, TRAF6, TRAF3, and IKKα-mediated response,thereby secreting large amounts of IFN-α. Similarly, the engagement of ligands in endosomescauses TLR8 initiate the MyD88-dependent pathway, culminating in synthesis and release of TNF-a and other proinflammatory mediators, via NF-κB activation.

IFN-α and TNF-α cooperatively mature myeloid dendritic cells. TLR7/8 agonist stimulates a specific population of inflammatory dermal dendritic cells referred as Tip-DCs to produce IL-23 after maturation by enhanced transcriptional activity.

Naive T cells differentiate into Naive Th17 by both IL-6 and TGF-β cells that express the transcription factors ROR-γt, ROR-α, and STAT3. These naive Th17 cells are self-activated by IL-21 in an autocrine manner and mature into Th17 cells which express IL-23 receptor on cell surface. Mature Th17 cells are activated by IL-23 stimulation. IL-23-mediated signal transduction produces cytokines IL-17.

IL-17 mediates the psoriasis response, promoting such activities as neutrophil migration to the epidermis,and proliferation of epidermal cells, which leads to the outbreak of psoriasis rash. Thus, psoriatic skin is induced mainly by overproduction of IL-17, which leads to a variety of adverse effects. We have identified a number of key events (KEs) along this pathway and created an AOP for stimulation of TLR7/8 that leads to psoriatic skin disease based on these key event relationships(KERs).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Sex Applicability

Sex	Evidence
Mixed	High

The proposed AOP for psoriasis-like skin thickening resulting from abnormal differentiation of keratinocytes, starting with Toll-like receptor (TLR) 7/8 activity, is independent of life stage, gender, or age (Lowes et al. 2007). The pathogenesis of psoriasis, an autoimmune disease, is genetically predisposed (3), but the autoantigen that causes psoriasis has not been identified (Zaba et al. 2008). Other causes of psoriasis are caused by external and internal triggers such as mild trauma, sunburn, infection, systemic drugs, and stress (Hansel et al. 2011). Stimulation of TLR7 / 8 releases INF-α and TNF-α in large amounts to produce IL-23, and Th17 cells mature by the stimulation to produce IL-17 and IL-22. In psoriasis skin formation, cytokines such as TNF-α, IL-23, and IL-17 work continuously. Since TNF-α inhibitors significantly suppressed IL-17A and IL-23p19 expression in psoriatic eruptions (Leonardi et al. 2012), by suppressing self-activation of Tip-DC by TNF-α, It can be seen that IL-23 and IL-17A production was suppressed. Anti-IL-17 and anti-IL-17RA antibodies suppress IL-17A and IL-17C, which are highly expressed in psoriatic eruptions. In particular, anti-IL-17RA antibody has been shown to normalize the expression of keratinocyte-related genes and IL-17C production two weeks after administration, followed by normalization of IL-17A production from leukocytes.

In mice, subcutaneous administration of IL-23 induced psoriatic eruption and IL-17A expression (K. A. et al. 2013), and IL-17C transgenic mice overexpressing IL-17C in keratinocytes showed psoriatic eruption. As shown in (8), the reaction of psoriasis-like eruption occurs in mice due to the chain of stimulation to T cells and epidermal cells starting from TLR.

Essentiality of the Key Events

Stressor, MIE and later events:MyD88 knock out(KO) mice

TLR7 (TLR7 / 8 in human) recognizes the imidazoquinoline derivative, binds to the adapter molecule MyD88, activates IRAKs (IL-1 receptor associated kinases), interacts with TRAF6 (TNF receptor associated factor 6) and IKK (Activates the IκB kinase complex). It phosphorylates IκB, induces its degradation, and transfers the transcription factor NF-κB to the nucleus. This pathway is called MyD88-dependent pathway and is essential for the production of inflammatory cytokines such as TNF-α (Akira S, Takeda K .: Nat Rev Immunol. Jul; 4: 499-511, 2004). When pDC is stimulated with a TLR7 / 8 ligand, the transcription factor IRF7 constitutively expressing pDC and MyD88 associate directly. IRF7 activity does not occur when pDCs of MyD88 KO mice are stimulated with TLR7 / 8 ligand. IRF7 is also activated by binding to TRAF6, leading to IFN-α production, which requires the Myd88 / TRAF6 / IRF7 complex. (Satoshi U, Shizuo A: Virus 54; 2: 145-152,2004)

Imiquimod 5% cream was applied to the left flank of female SKH-1 hairless mice (25 g body weight). The IFN-α and TNF-α concentrations in the skin after 1 and 2 hours of application increased these concentrations compared to the untreated skin.

In C57BL / 6 mice (8-12 weeks old) sensitized with 0.5% dinitrofluorobenzene (DNFB) as an antigen, imiquimod 5% cream was applied to the auricle once a day for 3 days. The application of imiquimod 5% cream promoted edema of the ears of mice (promoted DTH) compared to the base cream group. Imiquimod activates antigen-specific T cells by topical application to the skin. (Beserna Cream Interview Form Mochida Pharmaceutical Co., Ltd.)

KE-1 and later event:IL-17, IL-22 KO mice

In mice, psoriasis-like hyperplasia is induced by the application of IL-23, but this effect does not occur in IL-17A and IL-22 KO mice. IL-17A deficient mice show little epidermal hyperplasia after intradermal administration of IL-23. WT mice treated with anti-IL-17A Ab did not show IL-23-induced epidermal hyperplasia. IL-17 KO mice treated with IL-23 do not induce TNF-α mRNA and do not cause epidermal thickening. IL-22 did not increase in IL-17-/-mice after IL-23 administration, and IL-17 clearly increased in IL-22-/-mice. In IL-17-/-, IL-22-/-and WT mice treated with IL-23, immunohistochemically CD3 + T cells, CD11c (dendritic cells), F4 / 80 (macrophages), Gr-1 (Neutrophils) were analyzed. There was no difference in F4 / 80 and Gr-1 + cells in IL-17A-/-compared to WT mice, and CD3 + T cells decreased, but there was no obvious difference in IL-22-/-mice .

These data suggest that cytokines alone are not sufficient to mediate IL-23-induced epidermal changes, and that IL-17 and IL-22 are downstream mediators of mouse skin IL-23-induced changes. Therefore, Th17 cytokines are required for the generation of IL-23-mediated skin lesions.

KE-2 and later events: Mouse psoriasis-like dermatitis model

When TPA (12-O-tetradecanoy1phorbol-13-acetate) on the dorsal skin of K14 / mIL-1F6 gene-modified mice overexpress mouse IL-1F6 (IL-36a) selectively under the keratin 14 promoter was applied, skin pathological findings specific to psoriasis were observed, such as epidermal hyperplasia, epidermal exfoliation and micro-abscess formation, and wet inflammatory cells in the dermis. Quantitative RT-PCR measures mRNA expression levels of inflammatory chemokines and cytokines in skin tissues, and includes inflammatory chemokines: CCL3, CCL4, CXCL10,

CXCL1, and cytokines: IL-23, IL-12, IL-1β, etc. These expressions were elevated. (Kyowa Hakko Kirin Co., Ltd.)

References

Appendix 1

List of MIEs in this AOP

Event: 1706: Stimulation, TLR7/8 in dendritic cells (https://aopwiki.org/events/1706)

Short Name: Stimulation of TLR7/8

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:313 - Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease (https://aopwiki.org/aops/313)	MolecularInitiatingEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
dendritic cell

Organ term

Organ term
immune system

Domain of Applicability

Thirteen mammalian TLR members (10 in humans and 13 in mice) have been identified so far, of which TLR1, 2, 4, 5, and 6 are membrane bound and catalytic site for pathogenic structural components, whereas TLR3, 7, 8, and 9 expressed within the endosomal compartment

are dedicated to nucleic acids. TLRs 1–9 are conserved among humans and mice, yet TLR10 is present only in humans and TLR11 strictly restricted to rodents (Gupta et al. 2016).

Mouse TLR10 is not functional because of a retrovirus insertion, and TLR11, TLR12 and TLR13 have been lost from the human genome. (Kawai and Akira. 2010).

In addition, alignment of amino acid residues between human toll-like receptor 7 (AAF60188.1) and murine toll-like receptor 7 (AGX25544.1) was 80.74% identification. Both proteins have 1049 amino acid residues.

Structural characterization was conducted with recombinant TLR7 from monkey (*Macaca mulatta*; 96.8% sequence identify with human TLR7) expressed in *Drosophila* S2 cells (Zhang et al. 2016).

Studies of DC subsets isolated from humans and mice have revealed that TLRs have distinct expression patterns. Freshly isolated human pDCs express TLR7 and TLR9, whereas $CD11c^+$ human myeloid DCs (mDCs) express TLR1, TLR2, TLR3, TLR5, TLR6 and TLR8. In some studies, TLR7 expression was detected on both pDCs and mDCs, whereas others found TLR7 was exclusively expressed in pDCs (Iwasaki and Medzhitov. 2004).

In mice, all splenic DC subsets express TLRs 1, 2, 4, 6, 8 and 9. However, mouse pDCs do not express TLR3. Moreover, mouse $CD8\alpha^+$ DCs lack TLR5 and TLR7 expression and fail to respond to TLR7 agonists. In short, $CD4^+$ DC, CD4/CD8DN DC and pDC express TLR7 in mice (Iwasaki and Medzhitov. 2004).

Key Event Description

Toll-like receptors (TLRs) are members of interleukin-1 (IL-1) receptor/TLR superfamily, as they share the intracellularToll-IL-1 receptor (TIR) domain with the IL-1 receptor.

Toll-like receptor (TLR) 7 and TLR8 is known to mediate the recognition of guanosine- and uridine-rich single-stranded RNA (ssRNA) derived from ssRNA viruses and synthetic antiviral imidazoquinoline components (Akira et al. 2006, Blasius and Beutler. 2010). They also mediate the recognition of self RNA that is released from dead or dying cells.

Human TLR7 (hTLR7) and human TLR8 (hTLR8) contains 1049, 1041 amino acid residues with a calculated molecular weight of 120.9 kDa and 119.8 kDa respectively (Chuang and Ulvitch. 2000). The full-length hTLR7 protein includes a signal peptide of 26 amino acids (1–26 aa). The mature hTLR7 protein ectodomain, trans-membrane, and TIR domain are composite structure of 27–839, 840–860, and 889–1,036 amino acids, respectively (Gupta et al. 2016).

hTLR7 and hTLR8 form a subfamily of proteins that each contain an extracellular domain of >800 residues and share functional and structural features. TLR8 contains 26 leucine-rich repeats (LRRs), which is the largest number of LRRs among TLRs whose structures have been reported (Tanji et al. 2013).

Monkey TLR7 exists as a monomer in the absence of ligands, and TLR7 dimerization is induced by R848 alone, but not by poly U or guanosine alone, although these two ligands synergistically triggered TLR7 dimerization (Zhang et al. 2016). In contrast, hTLR8 exists as preformed dimer before ligand recognition. TLR8 is activated by R848 alone, or both uridine and ssRNA synergistically (Tanji et al. 2013).

The key residues interacting two TLR7 molecules into dimer confirmation are LYS410, ASN503, SER504, GLY526, ASN527, SER530, THR532, ARG553, and TYR579 (Gupta et al. 2016).

TLR3, TLR7, TLR8, and TLR9 localize to the endoplasmic reticulum and are trafficked to the endosomal compartment where they initiate cellular responses upon their activation by PAMPs and DAMPs (Lai et al. 2017).

TLR7 are exclusively expressed in plasmacytoid DCs (pDCs), which have the capacity to secrete vast amounts of type I IFN rapidly in response to viral infection (Gilliet et al. 2008, Reizis et al. 2011).

TLR8 is expressed in various tissues, with its highest expression in monocytes. Myeloid DCs (mDCs) also express TLR8 in human (Iwasaki and Medzhitov. 2004). Thus, TLR8 ligands can directly activate mDCs via TLR8.

TLR7-mediated signaling in pDC is mediated in a MyD88-dependent fashion, which initiates an IRF7-mediated response, secreting vast amounts of IFN type 1 (Kawai and Akira. 2011).

MyD88-dependent IRF7 activation in pDCs is mediated by activation of IRAK1, TRAF6, TRAF3, and IKKα and is facilitated by IFN-inducible Viperin expressed in the lipid body (Kawai and Akira. 2011).

IRF7, which is constitutively expressed by pDCs, binds MyD88 and forms a multiprotein signaling complex with IRAK4, TRAF6, TRAF3, IRAK1 and IKKα (Kawai and Akira. 2008). In this complex, IRF7 becomes phosphorylated by IRAK1 and/or IKKα, dissociates from the complex and translocates into the nucleus.

The interferons (IFNs) are a primary defense against pathogens because of the strong antiviral activities they induce. Three types of IFNs, types I, II and III, have been classified based on of their genetic, structural, and functional characteristics and their cell-surface receptors (Zhou et al. 2014). IFN-α belongs to the type I IFNs, the largest group which includes IFN-β, IFN-ε, IFN-ω, IFN-κ, IFN-δ, IFN-τ and IFN-ζ.

The IFN-α of type I IFN family in humans is composed of 12 subtypes encoded by 14 nonallelic genes including one pseudogene and two genes that encode the same protein. The various IFN-α subtypes have many common points. For example, all are clustered on chromosome 9 (Diaz et al. 1993). IFN-αs, which are composed of 165 to 166 aa, have 80% amino acid sequence identities (Li et al. 2018).

Upon engagement of ssRNAs in endosomes, TLR8 initiate the MyD88-dependent pathway culminating in synthesis and release of proinflammatory mediators, such as TNF-α via NF-κB activation (Tanji et al. 2015).

Stimulation of blood DCs with self-RNA–LL37 complexes induces a robust TNF-α response (Hänsel et al. 2011). DC activation is known to be enhanced by IFN-α in the presence of TNF-α (Luft et al. 1998).

How it is Measured or Detected

HEK293 cells were transiently co-transfected with human TLR7 and NF-κB-luciferase reporter. After 48 hours, the cells were stimulated with various concentrations of resiquimod or imiquimod. Luciferase activity was measured 48h post-stimulation and the results are reported as fold-increase in luciferase production relative to medium control (Gibson et al. 2002). R848 (0.001-10 μg/mL) induced NF-κB activation in HEK293 cells transfected with human TLR8 is detected in the same manner (Jurk et al. 2002).

IFN-α in cell-free supernatants collected after imidazoquinoline stimulation to human PBMC and/or pDC-enriched cells is detected by ELISA (Gibson et al. 2002).

TNF-α and IL-6 in cell-free supernatants collected after RNA-LL37 stimulation to mDCs were measured by ELISA (Ganguly et al. 2009). mDCs were also stained with fluorochrome-labeled anti-CD80, anti-CD86, and anti-CD83 antibodies and analyzed by flow cytometry (Ganguly et al. 2009).

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List of Key Events in the AOP

Event: 1707: Overproduction of IL-23, matured dendritic cells (https://aopwiki.org/events/1707)

Short Name: Overproduction of IL-23

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:313 - Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease (https://aopwiki.org/aops/313)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
dendritic cell

Organ term

Organ term
immune system

Domain of Applicability

pDCs were prepared from mouse spleen, and cytokine production after culture with IMQ was measured. IFN-a production from splenic pDCs was induced by IMQ treatment. The production of IL-23, IL-6 and TNF-α was also induced by IMQ treatment. Although 4–8% of mPDCA-1$^-$ CD11c$^+$ DCs were contaminated in prepared mPDCA-1$^+$ pDC fraction, we confirmed that splenic mPDCA-1$^-$ CD11c$^+$ DCs enriched to approximately 80% purity could not produce IL-23 and TNF-α by IMQ stimulation. In Tlr7-/- splenic pDCs, these cytokines (IFN-α, IL-23, IL-6 and TNF-α) were not induced by IMQ treatment, although stimulation by CpG, a TLR9 ligand, resulted in induction of these cytokines at the same level as was produced by wild-type splenic pDCs. These data indicate that, in mice, IMQ application can induce the production via TLR7 of IFN-a, IL-23, IL-6 and TNF-α from pDCs existing in the skin in vivo (Ueyama et al. 2014).

Key Event Description

A distinct population of human blood DCs that are defined by the selective expression of the 6-sulfo LacNAc residue on the P-selectin glycoprotein ligand 1 membrane molecule was described previously. 6-Sulfo LacNAc DCs (slanDCs) stand out by a marked production of TNF-α, and they were recognized as the major source of IL-12p70 among blood leukocytes when stimulated with CD40 ligand or LPS of gramnegative bacteria (Hänsel et al. 2011).

According to the current concept, these inflammatory DCs are CD1c$^-$, CD11c$^+$ cells locally expressing TNF-α and iNOS. They were also referred to as TNF and inducible nitric oxide synthase–expressing DCs (Tip-DCs) (Lowes et al. 2005) or inflammatory dermal DCs (Zaba et al. 2009). In contrast, resident dermal DCs express CD1c and CD11c and were shown to lack inflammatory markers. The phenotype of slanDCs (CD11c$^+$ and CD1c$^-$) and their local production of IL-23p19, TNF-α, and iNOS identify slanDCs as being a population of inflammatory dermal DCs and so-called Tip-DCs in psoriasis (Hänsel et al. 2011).

Stimulation of blood DCs with self-RNA–LL37 complexes induced a robust TNF-α response (Hänsel et al. 2011). TNF-α affects Tip-DCs in an autocrine and/or paracrine manner (Zaba et al. 2007).

DC activation is known to be enhanced by IFN-α in the presence of TNF-α (Luft et al. 1998).

R848 induces IL-23 production from activated human monocyte-derived DCs (moDCs) by enhanced transcriptional activity (Schwarz et al. 2013).

IL-23 is a heterodimer, sharing a p40 subunit with IL-12 but having a distinct p19 subunit. IL-23 binds to IL-12Rβ1 but not IL-12Rβ2. The receptor for this cytokine is heterodimeric and uses a novel second subunit, IL-23R, which is a member of the hematopoietin receptor family (Lee et al. 2004).

How it is Measured or Detected

IL-23 in cell-free supernatants collected after R848 stimulation to moDCs is detected by ELISA (Schwarz et al. 2013). Expression of IL-23 mRNA in R848-stimulated moDCs is measured by qRT-PCR (Schwarz et al. 2013).

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Event: 1708: Overproduction of IL-17 from Th17 cells (https://aopwiki.org/events/1708)

Short Name: Overproduction of IL-17

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:313 - Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease (https://aopwiki.org/aops/313)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
dendritic cell

Organ term

Organ term
immune system

Domain of Applicability

Ras homolog gene family H (RhoH) is a membrane-bound adapter protein involved in proximal T cell receptor signaling, and spontaneously develops chronic dermatitis that closely resembles human psoriasis in RhoH gene-deficient mice. Ubiquitin protein ligase E3 component N recognition 5 (Ubr5) and nuclear receptor subfamily 2 group F member 6 (Nr2f6) expression levels are decreased at the lesion site, and protein levels and DNA binding activity of retinoic acid-related orphan receptors are increased is doing. As a result, T cells differentiated into Th17 cells due to increased production of IL-17 and IL-22. These results indicate that RhoH suppresses the differentiation of naive T cells into effector Th17 cells. RhoH is a gene expressed in blood cells, and when RhoH expression decreases in T cells, Th17 cells increase, IL-22 is produced in large quantities, and the epidermis thickens, leading to the formation of psoriasis pathology. Humans with low RhoH expression may become more severe if they suffer from psoriasis. Journal of Allergy and Clinical Immunology

Key Event Description

Psoriasis has been known to play a major role in the pathogenesis of T cell dysfunction, particularly overactivation of the Th17 pathway. Th17 cells are a subset of CD4 positive T helper cells newly found in 2005 as a cell population different from Th1 and Th2 (Lisa C. et al. 2007).

Serum IL-17 levels in psoriasis patients are significantly higher than in healthy individuals, and the neutralizing antibody Brodalumab against the IL-17A receptor has been shown to be effective in treating psoriasis (Gilliet et al. 2004). Furthermore, because the antibody preparations against IL-17 (Ixéquizumab [John K. et al. 2002]), Sequinumab (Szeimies et al. 2004)) are used for the treatment of psoriasis, the Th17 pathway for pathogenesis is considered to play an important role.

Psoriatic CD4 and CD8 T cells infiltrate both the epidermis and dermis and show increased expression of IL17A, IL22, and IFNG in epidermal CD4 and CD8 T cells near keratinocytes, but dermal T cells Less up-regulation. Cheuk et al. 2013

IL-22, produced mainly by lesional epidermal CD4 T cells, is associated with the activation of keratinocytes and the formation of epidermal thickening, a prominent morphological feature of psoriasis. The lesional epidermal CD8 T cells mainly produce IL-17A and promote the production of inflammatory cytokines and chemokines by keratinocytes. IL-17A is an important mediator of psoriatic inflammation through the recruitment and activation of leukocytes to the skin.(Cheuk et al. 2013)

How it is Measured or Detected

Flow cytometric analysis of psoriatic skin biopsy showed increased frequency of IL-17 + and IL-22 + CD4 + T cells, and IL-17 secretion was significantly increased. CD4 + cells making IL-17 or IL-22 expressed IL-23R, and the frequency of IL-17 +, CCR6 + and CCR4 + T cells increased. The frequency of IL-17 + and IL-22 + CD4 + T cells was increased compared with normal skin, and the proportion of IL-22 positive IL-17 + cells was high. There was also an increase in IL-22 producing cells (Th-22 cells) that do not produce IL-17 or IFNγ. (Benham et al. 2013)

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List of Adverse Outcomes in this AOP

Event: 1709: Psoriatic skin disease (https://aopwiki.org/events/1709)

Short Name: Skin disease

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:313 - Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease (https://aopwiki.org/aops/313)	AdverseOutcome

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Mouse psoriasis-like dermatitis model: K14 / mIL-1F6 gene-modified mice overexpress mouse IL-1F6 (IL-36a) selectively under the keratin 14 promoter, and TPA: 12-O- tetradecanoy1phorbol-13-acetate(TPA) was applied, skin pathological featuresfindings specific to psoriasis-such as epidermal hyperplasia, epidemal exfoliation and micro-abscess formation, and wet inflammatory cells in the dermis-were observed. Quantitative RT-PCR. Measures mRNA expression levels of Inflammatory chemokines and cytokines in skin tissues, and includes inflammatory chemokines: CCL3, CCL4, CXCL10, CXCL1 and cytokines: IL-23, IL-12, IL-1β etc. Expression was observed. (Kyowa Hakko Kirin Co., Ltd.)

Key Event Description

In psoriasis vulgaris, the S100 protein family psoriasin (sorazine) and koebnerisin (kebneridine) are overexpressed, and the epidermal antimicrobial peptide induced by IL-17 functions itself as a chemotactic factor and cytokine. It recruits CD4 + T cells and neutrophils to exacerbate inflammation. (Kanagawa Psoriasis Treatment Study Group)

A biopsy of the skin area of psoriasis and surrounding normal skin was performed, and immunohistological examination of the sections was performed. In psoriatic lesions, the number of activated dendritic cells was increased, and CD1a-positive Langerhans cells in the epidermis and CD83-positive CD1a-negative Langerin-negative CD11c-positive dermal dendritic cells in the epidermis boundary were observed. In normal skin,

the number of wet cells was the same as in the lesion, but CD3-positive T lymphocytes were significantly less than in the lesion.

In the normal area, inflammatory keratin K6 and K16-positive keratinocytes were found, and the transcription factor C / EBPβ, which is normally expressed only in the granular layer of the normal epidermis, was expressed in the entire epidermis as in the lesion. This suggests that early inflammatory changes have already occurred in normal areas that have not yet shown obvious skin lesions, and that these changes are caused by dendritic cells rather than lymphocytes. (Komine et al. 2007)

How it is Measured or Detected

A biopsy of the skin area and the surrounding normal skin of a patient with psoriasis vulgaris was performed, and immunohistological studies were performed using dendritic cell surface markers and lymphocyte surface markers as primary antibodies in the sections. In the vicinity of the psoriatic lesion, an increased number of activated dendritic cells was observed, and CD1a-positive Langerhans cells in the epidermis and CD83-positive CD1a-negative Langerin-negative CD11c-positive dermal dendritic cells in the epidermis boundary were observed. In normal skin, the number of wet cells was the same as in the lesion, but CD3-positive T lymphocytes were significantly less than in the lesion. In the normal area, inflammatory keratin K6 and K16-positive keratinocytes were found, and the transcription factor C / EBPβ, which is normally expressed only in the granular layer of the normal epidermis, was expressed in the entire epidermis as in the lesion. This suggests that early inflammatory changes have already occurred in normal areas that have not yet shown obvious skin lesions, and that these changes are caused by dendritic cells rather than lymphocytes. (Komine et al..2007)

Serum amyloid A: SAA was measured in 35 psoriasis patients and healthy humans. DNA microarray analysis in lesions of psoriasis patients showed that SAA levels were about 5 times higher than normal skin. The average SAA in psoriasis patients was 19.1 ug / ml, and the SAA after treatment was significantly reduced to an average of 6.9 ug / ml. There was a correlation between SAA and psoriasis severity score (PASI). In addition, amyloid A deposition, which is thought to be the result of prolonged chronic inflammation, was observed in the skin-stained section of the psoriatic lesion skin area. (J Dermatolog Treat. 2013; 24 (6): 477-80)
Epidermal keratinocyte expression genes that were elevated in psoriatic lesions of patients with psoriasis with stage-type skin eruption: mRNA expression level of keratin6a and 16, s100A7A, S100A12, DEFB4, IL-1F6, CCL20, IL-17C, etc. was rapidly reduced by 700 single intravenous dose of brodalumab and decreased to non-lesional skin level two weeks after administration.On the other hand, leukocyte expression genes with increased expression in psoriatic lesion skin: IL-17A, IL-17F, IL-23F, IL-12B, IL-22, IFN-γ and other mRNA expression levels decreased with brodalumab administration However, at 2 weeks after administration, the level did not decrease to the level of the non-lesional skin. Since the expression of pathophysiology-related genes is reduced prior to the decrease in the expression of leukocyte expression genes and the decrease in the PASI score, brodalumab expresses pathophysiology-related genes by blocking IL-17 signaling in the epidermal keratinocytes of psoriatic lesions It is possible to improve the skin eruption promptly. (Kyowa Hakko Kirin Co., Ltd.)

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2017: Stimulation of TLR7/8 leads to Overproduction of IL-23 (https://aopwiki.org/relationships/2017)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease (https://aopwiki.org/aops/313)	**adjacent**	**High**	**High**

Evidence Supporting Applicability of this Relationship

Thirteen mammalian TLR members (10 in humans and 13 in mice) have been identified so far, of which TLR1, 2, 4, 5, and 6 are membrane bound and catalytic site for pathogenic structural components, whereas TLR3, 7, 8, and 9 expressed within the endosomal compartment

are dedicated to nucleic acids. TLRs 1–9 are conserved among humans and mice, yet TLR10 is present only in humans and TLR11 strictly restricted to rodents (Gupta et al. 2016).

Mouse TLR10 is not functional because of a retrovirus insertion, and TLR11, TLR12 and TLR13 have been lost from the human genome (Kawai and Akira. 2010).

In addition, alignment of amino acid residues between human toll-like receptor 7 (AAF60188.1) and murine toll-like receptor 7 (AGX25544.1) was 80.74% identification. Both proteins have 1049 amino acid residues.

Structural characterization was conducted with recombinant TLR7 from monkey (*Macaca mulatta*; 96.8% sequence identify with human TLR7) expressed in *Drosophila* S2 cells (Zhang et al. 2016).

Studies of DC subsets isolated from humans and mice have revealed that TLRs have distinct expression patterns. Freshly isolated human pDCs express TLR7 and TLR9, whereas $CD11c^+$ human myeloid DCs (mDCs) express TLR1, TLR2, TLR3, TLR5, TLR6 and TLR8. In some studies, TLR7 expression was detected on both pDCs and mDCs, whereas others found TLR7 was exclusively expressed in pDCs (Iwasaki and Medzhitov. 2004).

In mice, all splenic DC subsets express TLRs 1, 2, 4, 6, 8 and 9. However, mouse pDCs do not express TLR3. Moreover, mouse $CD8\alpha^+$ DCs lack TLR5 and TLR7 expression and fail to respond to TLR7 agonists. In short, $CD4^+$ DC, CD4CD8DN DC and pDC express TLR7 in mice (Iwasaki and Medzhitov. 2004).

Although unpublished, it has been reported that human slanDCs (Tip-DCs) lack the DNA-binding structureTLR9 but can express the endosomal RNA-binding receptorsTLR8 (slanDCs and$CD1c^+$ DCs) and TLR7 (slanDCs but not $CD1c^+$ DCs; Hänsel et al, unpublished data, June 2010) (Hänsel et al. 2011). There are not any other reports which mentioned TLR7 expression in Tip-DCs, so whether or not TLR7 exists in human Tip-DCs is still unknown.

IFN-α, but not TNF-α and IL-6 production by human pDCs after stimulation with self-RNA-LL37 complex was detected (Ganguly et al. 2009). However, in mice, IFN-α production from splenic pDCs was induced by IMQ treatment. The production of TNF-α and IL-23 was also induced by IMQ treatment. Although 4–8% of mPDCA-1^- $CD11c^+$ DCs were contaminated in prepared mPDCA-1^+ pDC fraction, it was confirmed that splenic mPDCA-1^- $CD11c^+$ DCs enriched to approximately 80% purity could not produce TNF-α and IL-23 by IMQ stimulation. In $Tlr7^{-/-}$ splenic pDCs, these cytokines (IFN-α, TNF-α and IL-23) were not induced by IMQ treatment, although stimulation by CpG, a TLR9 ligand, resulted in induction of these cytokines at the same level as was produced by wild-type splenic pDCs. These data indicate that, in mice, IMQ application can induce the production via TLR7 of IFN-α, TNF-α and IL-23 from pDCs existing in the skin in vivo (Ueyama et al. 2014).

When BMDCs were generated by 10-day culture with GM-CSF and IL-4 and characterized their phenotypes, CD11c+ BMDCs showed MHC II^{high}, $CD11b^{high}$, $B220^-$, $CD86^{high}$, Mac-3^+, and had the ability to produce high levels of TNF-α and NO/iNOS in response to LPS stimulation, which represents a similar phenotype to Tip-DCs (Xu et al. 2007, Ueyama et al. 2014).

In these BMDCs which represents a similar phenotype to Tip-DCs, IMQ weakly but significantly induced the production of IL-23. In addition, although IFN-α had no effect alone, co-stimulation with IFN-α and IMQ resulted in marked induction of IL-23 production. However, using BMDCs derived from $Tlr7^{-/-}$ mice, these effects of IMQ and IFN-α was not observed, verifying that it is also mediated via TLR7 (Ueyama et al. 2014).

In mice, purified bone marrow dendritic cells (BMDCs) derived from wild-type mice stimulated with IFN-α showed increase in Tlr7 mRNA expression (Ueyama et al. 2014). In addition, TLR7 expression was also observed in the inflamed skin of IMQ-treated mice (Ueyama et al. 2014). These data suggest that the synergistic effect of IMQ and IFN-α on BMDCs was caused by induction of TLR7 expression by IFN-α (Ueyama et al. 2014).

Taken together, in mice, IFN-α produced by IMQ-primed pDCs may enhance the effects of IMQ to activate Tip-DC, resulting in the release of a large amount of IL-23 in IMQ-induced psoriasis-like skin lesion (Ueyama et al. 2014).

Key Event Relationship Description

Toll-like receptors (TLRs) are members of interleukin-1 (IL-1) receptor/TLR superfamily, as they share the intracellularToll-IL-1 receptor (TIR) domain with the IL-1 receptor.

Toll-like receptor (TLR) 7 and TLR8 is known to mediate the recognition of guanosine- and uridine-rich single-stranded RNA (ssRNA) derived from ssRNA viruses and synthetic antiviral imidazoquinoline components (Akira et al. 2006; Blasius and Beutler. 2010). They also mediate the recognition of self RNA that is released from dead or dying cells.

Human TLR7 (hTLR7) and human TLR8 (hTLR8) contains 1049, 1041 amino acid residues with a calculated molecular weight of 120.9 kDa and 119.8 kDa respectively (Chuang and Ulvitch. 2000).

The full-length hTLR7 protein includes a signal peptide of 26 amino acids (1–26 aa). The mature hTLR7 protein ectodomain, trans-membrane, and TIR domain are composite structure of 27–839, 840–860, and 889–1,036 amino acids, respectively (Gupta et al. 2016).

hTLR7 and hTLR8 form a subfamily of proteins that each contain an extracellular domain of >800 residues and share functional and structural features. TLR8 contains 26 leucine-rich repeats (LRRs), which is the largest number of LRRs among TLRs whose structures have been reported (Tanji et al. 2013).

Monkey TLR7 exists as a monomer in the absence of ligands, and TLR7 dimerization is induced by R848 alone, but not by poly U or guanosine alone, although these two ligands synergistically triggered TLR7 dimerization (Zhang et al. 2016). In contrast, hTLR8 exists as preformed dimer before ligand recognition. TLR8 is activated by R848 alone, or both uridine and ssRNA synergistically (Tanji et al. 2013).

The key residues interacting two TLR7 molecules into dimer confirmation are LYS410, ASN503, SER504, GLY526, ASN527, SER530, THR532, ARG553, and TYR579 (Gupta et al. 2016).

TLR3, TLR7, TLR8, and TLR9 localize to the endoplasmic reticulum and are trafficked to the endosomal compartment where they initiate cellular responses upon their activation by PAMPs and DAMPs (Lai et al. 2017).

TLR7 are exclusively expressed in plasmacytoid DCs (pDCs), which have the capacity to secrete vast amounts of type I IFN rapidly in response to viral infection (Gilliet et al. 2008, Reizis et al. 2011).

TLR8 is expressed in various tissues, with its highest expression in monocytes. Myeloid DCs (mDCs) also express TLR8 in human (Iwasaki and Medzhitov. 2004). Thus, TLR8 ligands can directly activate mDCs via TLR8.

TLR7-mediated signaling in pDC is mediated in a MyD88-dependent fashion, which initiates an IRF7-mediated response, secreting vast amounts of IFN type 1 (Kawai and Akira. 2011).

MyD88-dependent IRF7 activation in pDCs is mediated by activation of IRAK1, TRAF6, TRAF3, and IKKα and is facilitated by IFN-inducible Viperin expressed in the lipid body (Kawai and Akira. 2011).

IRF7, which is constitutively expressed by pDCs, binds MyD88 and forms a multiprotein signaling complex with IRAK4, TRAF6, TRAF3, IRAK1 and IKKα (Kawai and Akira. 2008). In this complex, IRF7 becomes phosphorylated by IRAK1 and/or IKKα, dissociates from the complex and translocates into the nucleus.

The interferons (IFNs) are a primary defense against pathogens because of the strong antiviral activities they induce. Three types of IFNs, types I, II and III, have been classified based on of their genetic, structural, and functional characteristics and their cell-surface receptors (Zhou et al. 2014). IFN-α belongs to the type I IFNs, the largest group which includes IFN-β, IFN-ε, IFN-ω, IFN-κ, IFN-δ, IFN-τ and IFN-ζ.

The IFN-α of type I IFN family in humans is composed of 12 subtypes encoded by 14 nonallelic genes including one pseudogene and two genes that encode the same protein. The various IFN-α subtypes have many common points. For example, all are clustered on chromosome 9 (Diaz et al. 1993). IFN-αs, which are composed of 165 to 166 aa, have 80% amino acid sequence identities (Li et al. 2018).

Upon engagement of ssRNAs in endosomes, TLR8 initiate the MyD88-dependent pathway culminating in synthesis and release of proinflammatory mediators, such as TNF-α via NF-κB activation (Tanji et al. 2015).

A distinct population of human blood DCs that are defined by the selective expression of the 6-sulfo LacNAc residue on the P-selectin glycoprotein ligand 1 membrane molecule was described previously. 6-Sulfo LacNAc DCs (slanDCs) stand out by a marked production of TNF-α, and they were recognized as the major source of IL-12p70 among blood leukocytes when stimulated with CD40 ligand or LPS of gramnegative bacteria (Hänsel et al. 2011).

According to the current concept, these inflammatory DCs are $CD1c^{-}$, $CD11c^{+}$ cells locally expressing TNF-α and iNOS. They were also referred to as TNF and inducible nitric oxide synthase–expressing DCs (Tip-DCs) (Lowes et al. 2005) or inflammatory dermal DCs (Zaba et al. 2009). In contrast, resident dermal DCs express CD1c and CD11c and were shown to lack inflammatory markers. The phenotype of slanDCs ($CD11c^{+}$ and $CD1c^{-}$) and their local production of IL-23p19, TNF-α, and iNOS identify slanDCs as being a population of inflammatory dermal DCs and so-called Tip-DCs in psoriasis (Hänsel et al. 2011).

Stimulation of blood DCs with self-RNA–LL37 complexes induced a robust TNF-α response (Hänsel et al. 2011). TNF-α affects Tip-DCs in an autocrine and/or paracrine manner (Zaba et al. 2007).

DC activation is known to be enhanced by IFN-α in the presence of TNF-α (Luft et al. 1998).

R848 induces IL-23 production from activated human monocyte-derived DCs (moDCs) by enhanced transcriptional activity (Schwarz et al. 2013).

IL-23 is a heterodimer, sharing a p40 subunit with IL-12 but having a distinct p19 subunit. IL-23 binds to IL-12Rβ1 but not IL-12Rβ2. The receptor for this cytokine is heterodimeric and uses a novel second subunit, IL-23R, which is a member of the hematopoietin receptor family (Lee et al. 2004).

Evidence Supporting this KER

Biological Plausibility

The molecular structure and function of TLR7 and TLR8 are evident based on sufficient scientific findings as mentioned above. The known mechanisms for stimulation of TLR7/8 by each ligand are initiated by the formation of homodimer. TLR7-mediated signaling in pDC is mediated in a MyD88-dependent fashion, which initiates an IRF7, IRAK1, TRAF6, TRAF3, and IKKα-mediated response, secreting vast amounts of IFN type 1 (Kawai and Akira. 2011).

Similarly, upon engagement of ligands in endosomes, TLR8 initiate the MyD88-dependent pathway culminating in synthesis and release of proinflammatory mediators, such as TNF-α via NF-κB activation (Tanji et al. 2015).

DC activation is known to be enhanced by IFN-α in the presence of TNF-α (Luft et al. 1998).

R848 induces IL-23 production from activated human monocyte-derived DCs (moDCs) by enhanced transcriptional activity (Schwarz et al. 2013).

TNF and inducible nitric oxide synthase–expressing DCs also known as Tip-DCs or inflammatory dermal DCs differentiates from moDCs by inflammation (Hänsel et al. 2011).

As mentioned above, stimulation of TLR7 in pDCs, and TLR8 in moDCs and Tip-DCs leads to activation of Tip-DCs, which leads to the overproduction of IL-23 from matured Tip-DCs.

Empirical Evidence

Much experimental data is available that supports the stimulation of TLR7 in pDC induced by TLR7 agonist, which subsequently promote secretion of IFN-α in MyD88-dependent fashion. For example, three populations of cells were evaluated for type I IFN production following imidazoquinoline stimulation: human PBMC, pDC-depleted PBMC, and pDC-enriched cells. Human PBMC produce IFN-α following imiquimod (0.3–30 μM) or resiquimod (0.03–30 μM) treatment. Peak levels of IFN-α were reached with imiquimod and resiquimod at 3 μM. PBMC, depleted of pDC, did not produce detectable levels of IFN-α in response to imiquimod or resiquimod treatment.

The imidazoquinoline-treated pDC-enriched cultures produced 2–20 times more IFN-α than similarly treated PBMC as measured over the entire dose range. Peak levels of Resiquimod- and imiquimod-induced IFN-α production were reached with 0.3 μM and 30 μM, respectively (Gibson et al. 2002).

In addition, pDCs were stimulated with LL37 premixed with total human RNA extracted from U937 cells to confirm that LL37 can interact with self-RNA and convert it into a trigger of IFN-α production. U937-derived self-RNA induced dose-dependent IFN-α production when mixed with LL37, but not when given alone or mixed with the scrambled peptide GL37. Similar to self-DNA (Lande et al., 2007), pDCs activated by self-RNA mixed with LL37 produced high levels of IFN-α, but did not produce TNF-α or IL-6 or undergo maturation as assessed by measuring the expression of costimulatory molecules CD80 and CD86 (Ganguly et al. 2009).

Importantly, self-RNA isolated from a variety of cell types and tissue samples from various types of skin pathologies induced similar levels of IFN-α when mixed with LL37, indicating that cellular- or disease-dependent variations in RNA composition do not play a role in the responses to self-RNA. These data demonstrate that LL37 can convert otherwise nonstimulatory self-RNA into a trigger of pDC activation to produce IFN-α, and thus enable the RNA released during cell death to induce innate immune activation (Ganguly et al. 2009).

IFN-α induced in pDCs by self-RNA–LL37 complexes was inhibited in a dose-dependent manner by bafilomycin, which blocks endosomal acidification and TLR signaling. To specifically inhibit TLR7, we used the short oligonucleotide C661, which selectively blocks TLR7 (Barrat et al. 2005), as shown by the inhibition of IFN-α induction by the synthetic TLR7 agonist R837 but not the TLR9 agonist CpG2006. Pretreatment of pDCs with C661 specifically blocked the IFN-α induction by self-RNA–LL37 complexes, indicating that pDC activation by self-RNA–LL37 complexes occurs through TLR7 (Ganguly et al. 2009).

Self-RNA–LL37 complexes but not self-RNA alone activated mDCs to produce the proinflammatory cytokines TNF-α and IL-6, but not IFN-α (Ganguly et al. 2009). Self-RNA–LL37 complexes also activated mDCs to undergo maturation as shown by the up-regulation of CD80 and CD86 expression (Ganguly et al. 2009) . mDC activation by self-RNA–LL37 complexes was entirely dependent on self-RNA, given that these responses were abrogated by decreasing the amount of self-RNA in the complexes (unpublished data). In contrast to self-RNA–LL37 complexes, self-DNA–LL37 complexes were unable to activate mDCs (Ganguly et al. 2009) . In accordance with these findings, stimulation of mDCs with supernatants of apoptotic cells combined with LL37 induced the secretion of proinflammatory cytokines, and this secretion was entirely dependent on self-RNA because activity was abolished by depletion of self-RNA but not self-DNA (Ganguly et al. 2009).

Compared with stimulation with either supernatant of activated pDCs or self-RNA–LL37 alone, the combination of both significantly enhanced the activation of mDCs to secrete IL-6 and TNF-α and enhanced their differentiation into mature CD83+ DCs (Ganguly et al. 2009). This activity was completely blocked by antibodies against IFN-α, IFN-β and IFN-αβR (Ganguly et al. 2009). Thus, self-RNA–LL37 complexes can trigger mDC activation and maturation, and this process is enhanced by the concomitant activation of pDCs to produce IFN-α.

Self-RNA was also internalized by mDCs when complexed with LL37 but not when given alone. The cytokine production such as TNF-α and IL-6 of mDCs induced by self-RNA–LL37 complexes but not by the TLR4 agonist LPS was completely inhibited by bafilomycin in a dose-dependent manner, demonstrating that mDC activation by self-RNA–LL37 complexes involved endosomal TLR activation. Using 293T cells transfected with TLR8 and TLR3 expression vectors along with a NF-κB luciferase reporter plasmid, it was confirmed that self-RNA–LL37 complexes activated TLR8 but not TLR3. In support of this finding, synthetic short ssRNA sequences that activate TLR8 in human mDCs (Diebold et al. 2004, Heil et al. 2004) also activated mDCs when complexed with LL37 but not when given alone (Ganguly et al. 2009).

Dose-dependent DC maturation was observed with increasing concentrations from 10 IU/ml up to 1000 IU/ml of IFN-α2a or IFN-α8 added to cultures containing GM-CSF, IL-4, and TNF-α. Both of the IFNs had a similar capacity to up-regulate HLA-A, B, C, CD80, and CD86 and to down-regulate CD1a and CD11b expression on the cell population (Luft et al. 1998).

DC cultured in GM-CSF, TNF-α, and IL-4-containing medium until day 14, and type I IFNs were added daily between days 14 and 17. Proportions of positive cells for each markers were analyzed by FACS on day 17 (Luft et al. 1998).

When GM-CSF, TNF-α, and IL-4-containing cultures were washed on day 14 and continued until day 17 in serum-free medium containing GM-CSF and IL-4, without or with TNF-α (20 ng/ml, standard conditions), IFN-a (1000 IU/ml), or both, IFN-α alone did not enhance DC maturation as compared with standard conditions. When both of TNF-α and IFN-α exist, optimal maturation was observed than either TNF-α or IFN-α alone. Thus, the enhancement of DC activation by IFN-α under serum-free conditions required the presence of TNF-α (Luft et al. 1998).

LL37 is highly expressed in the inflamed skin of psoriasis but is undetectable in inflamed skin of atopic dermatitis or in healthy skin (Lande et al. 2007). To determine whether extracellular self-RNA–LL37 complexes form in vivo, Staining skin sections with Ribogreen and DAPI revealed that numerous extracellular Ribogreen^{+}/ DAPI^{-} complexes in the dermal compartment of psoriatic skin lesions, but not in skin of atopic dermatitis or healthy skin (Ganguly et al. 2009). These tissue RNA complexes presented several features of self-RNA–LL37 complexes generated in vitro, including the size and bead-like branched structures resulting from the aggregation of smaller particles (Ganguly et al. 2009).

Skin sections of psoriatic tissues were stained with an anti-LL37 antibody and Ribogreen to determine whether the self-RNA complexes in the tissues contained LL37 and it was found that the majority of these complexes contained LL37 (Ganguly et al. 2009). Importantly, psoriatic skin also contained substantial numbers of particulate self-DNA–LL37 complexes.

Serial sections of lesional psoriatic skin were stained for RNA complexes and DC-LAMP, a lysosomal marker specific for mature mDCs to determine whether the presence of tissue self-RNA complexes is associated with the presence of activated DCs in psoriatic skin. Consistent with previous reports (Lowes et al. 2005), it was found that large clusters of DC-LAMP–positive mature mDCs (Ganguly et al. 2009). We also found tissue self-RNA–LL37 complexes within these clusters, and, occasionally, even inside the DCs as shown by the colocalization with endolysosomal compartments stained with DC-LAMP (Ganguly et al. 2009). The number of tissue self-RNA complexes significantly correlated with the numbers of DC-LAMP–positive mDCs in psoriatic skin (Ganguly et al. 2009). Together, these findings strongly support *in vitro* data that self-RNA complexes can activate mDCs and suggest that this pathway is operational in psoriasis.

When mRNA expression normalized to HARP for IL-23 subunits, such as p19 and p40 were quantified by RT-PCR in monocyte-derived DCs (moDCs) matured without and with etanercept, a dimeric human tumor necrosis factor recepter p75-Fc fusion protein made of 2 extra-cellular domains of the human 75kD TNFR linked by the constant Fc portion of human IgG1 (Haraoui and Bykerk. 2007), significant decrease in expression of IL-23 subunits p19 and p40 by etanercept were observed (Zaba et al. 2007). MoDCs cultured with etanercept decreased CD86

expression threefold and HLA-DR expression fivefold. In addition, moDCs cultured with etanercept were also an average of two to threefold less stimulatory than control DCs in a mixed leukocyte reaction. Gene array on control moDCs compared with those cultured with etanercept revealed that CD163, a macrophage scavenger receptor, was up-regulated 6.5-fold (Zaba et al. 2007).

In psoriatic dermis, mRNA expression normalized to HARP for multiple inflammatory products of Tip-DCs, including iNOS, TNF-α and IL23 p40 subunit, are reduced within 1–2 weeks after beginning etanercept, whereas the number of $CD11c^+$ DCs in the tissue is minimally affected during this time, suggesting an initial blockade of cytokine production by these cells rather than cell reduction (Zaba et al. 2007). These facts suggest that TNF-α is an autocrine or paracrine inducer of IL-23 from Tip-DC (Zaba et al. 2007).

R848-treatment to moDCs, which were generated from monocytes isolated from buffy coats of healthy donors, resulted in concentration-dependent expression of IL-23. 2×10^5 moDCs/ml were plated in DC medium and stimulated with 0-5 μg/ml R848. After 24 h of TLR stimulation, supernatants were harvested and cytokine expression was measured by ELISA. In addition, the combination of NOD1 and NOD2 agonists with R848 stimulated high levels of IL-23 secretion (Schwarz et al. 2013).

qRT-PCR for moDCs stimulated with TLR agonists in the absence or presence of NOD1 and NOD2 ligands at 8 h and 24 h post induction revealed that synergistic cytokine expression observed in NOD1/NOD2- and R848-stimulated cells is largely mediated by enhanced transcriptional activity (Schwarz et al. 2013).

In time kinetic studies, moDCs were stimulated with R848 in the absence or presence of MDP and iE-DAP which are ligands of NOD1/2, for 30 min, 2 h, 8 h or 24 h and mRNA levels of IL-23 as well as the cumulative cytokine release were measured by qRT-PCR and sandwich-ELISA, respectively. At the mRNA level, synergistic effects of both NOD ligands with R848 are already detectable after 8 h of stimulation. In agreement with IL-23 mRNA expression, synergistic effects are detectable by ELISA after 8 h; nevertheless, these effects are even more pronounced after 24 h of stimulation (Schwarz et al. 2013).

These findings show that dose responses and temporality of MIE and KE1 seem to be in sequence.

Uncertainties and Inconsistencies

Although unpublished, it has been reported that human slanDCs (Tip-DCs) lack the DNA-binding structureTLR9 but can express the endosomal RNA-binding receptorsTLR8 (slanDCs and$CD1c^+$ DCs) and TLR7 (slanDCs but not $CD1c^+$ DCs; Hänsel et al, unpublished data, June 2010) (Hänsel et al. 2011). There are not any other reports which mentioned TLR7 expression in Tip-DCs, so whether or not TLR7 exists in human Tip-DCs is still unknown.

In addition, freshly isolated human pDCs have been reported to express TLR7 and TLR9, whereas $CD11c^+$ human myeloid DCs (mDCs) express TLR1, TLR2, TLR3, TLR5, TLR6 and TLR8. In some studies, TLR7 expression was detected on both pDCs and mDCs, whereas others found TLR7 was exclusively expressed in pDCs. Therefore, it is still unknown that whether or not TLR7 exists in human mDCs, and how much it does contribute recognition of R848 or LL37-RNA in these cells (Iwasaki and Medzhitov. 2004).

Quantitative Understanding of the Linkage

Response-response relationship

MIE:

Much experimental data is available that supports the stimulation of TLR7 in pDC induced by TLR7 agonist, which subsequently promote secretion of IFN-α in MyD88-dependent fashion. For example, HEK293 cells were transiently co-transfected with human TLR7 and NF-κB-luciferase reporter. After 48 hours, the cells were stimulated with various concentrations of resiquimod or imiquimod. Luciferase activity was measured 48h post-stimulation and the results are reported as fold-increase relative to medium control. As a result, dose-dependent increase in NF-κB-dependent luciferase activity in HEK293 transfected with hTLR7 was observed with increasing concentrations from 0.01 μM up to 10 μM of resiquimod, and 0.1 μM up to 15 μM of imiquimod. Maximal NF-κB activation with resiquimod is achieved with 10-30 μM, which yields an 18-fold increase in luciferase production. Maximal NF-κB activation with imiquimod requires 10-15 μM compound and induces a 5-6-fold increase in luciferase production (Gibson et al. 2002).

In addition, three populations of cells were evaluated for type I IFN production following imidazoquinoline stimulation: human PBMC, pDC-depleted PBMC, and pDC-enriched cells. Human PBMC produce IFN-α following imiquimod (0.3–30 μM) or resiquimod (0.03–30 μM) treatment. Peak levels of IFN-α were reached with imiquimod and resiquimod at 3 μM. PBMC, depleted of pDC, did not produce detectable levels of IFN-α in response to imiquimod or resiquimod treatment.

The imidazoquinoline-treated pDC-enriched cultures produced 2–20 times more IFN-α than similarly treated PBMC as measured over the entire dose range. Peak levels of Resiquimod- and imiquimod-induced IFN-α production were reached with 0.3 μM and 30 μM, respectively (Gibson et al. 2002).

In different experiments, pDCs were stimulated with LL37 premixed with total human RNA extracted from U937 cells to confirm that LL37 can interact with self-RNA and convert it into a trigger of IFN-α production. U937-derived self-RNA induced dose-dependent IFN-α production when mixed with LL37, but not when given alone or mixed with the scrambled peptide GL37 (Ganguly et al. 2009).

R848 (0.001-10 μg/mL) induced NF-κB activation in HEK293 cells transfected with human TLR8 in a dose-dependent manner (Jurk et al. 2002). In addition, the production of TNF-α and IL-6, and the maturation

of mDCs induced by self-RNA–LL37 complexes but not by the TLR4 agonist LPS was completely inhibited by bafilomycin in a dose-dependent manner, demonstrating that mDC activation by self-RNA–LL37 complexes involved endosomal TLR activation (Ganguly et al. 2009).

Dose-dependent DC maturation was observed with increasing concentrations from 10 IU/ml up to 1000 IU/ml of IFN-α2a or IFN-α8 added to cultures containing GM-CSF, IL-4, and TNF-α. Both of the IFNs had a similar capacity to up-regulate HLA-A, B, C, CD80, and CD86 and to down-regulate CD1a and CD11b expression on the cell population (Luft et al. 1998).

DC cultured in GM-CSF, TNF-α, and IL-4-containing medium until day 14, and type I IFNs were added daily between days 14 and 17. Proportions of positive cells for each markers were analyzed by FACS on day 17 (Luft et al. 1998).

When GM-CSF, TNF-α, and IL-4-containing cultures were washed on day 14 and continued until day 17 in serum-free medium containing GM-CSF and IL-4, without or with TNF-α (20 ng/ml, standard conditions), IFN-a (1000 IU/ml), or both, IFN-α alone did not enhance DC maturation as compared with standard conditions. When both of TNF-α and IFN-α exist, optimal maturation was observed than either TNF-α or IFN-α alone. Thus, the enhancement of DC activation by IFN-α under serum-free conditions required the presence of TNF-α (Luft et al. 1998).

In accordance with these findings, compared with stimulation with either supernatant of activated pDCs or self-RNA–LL37 alone, the combination of both significantly enhanced the activation of mDCs to secrete IL-6 and TNF-α and enhanced their differentiation into mature CD83+ DCs (Ganguly et al. 2009). This activity was completely blocked by antibodies against IFN-α, IFN-β and IFN-αβR (Ganguly et al. 2009). Thus, self-RNA–LL37 complexes can trigger mDC activation and maturation, and this process is enhanced by the concomitant activation of pDCs to produce IFN-α.

KE 1

R848-treatment to moDCs, which were generated from monocytes isolated from buffy coats of healthy donors, resulted in concentration-dependent expression of IL-23. 2×10^5 moDCs/ml were plated in DC medium and stimulated with 0-5 µg/ml R848. After 24 h of TLR stimulation, supernatants were harvested and cytokine expression was measured by ELISA. In addition, the combination of NOD1 and NOD2 agonists with R848 stimulated high levels of IL-23 secretion (Schwarz et al. 2013).

qRT-PCR for moDCs stimulated with TLR agonists in the absence or presence of NOD1 and NOD2 ligands at 8 h and 24 h post induction revealed that synergistic cytokine expression observed in NOD1/NOD2- and R848-stimulated cells is largely mediated by enhanced transcriptional activity (Schwarz et al. 2013).

Time-scale
Human PBMC, pDC-deficient PBMC, and pDC -enriched from human PBMC (pDC-enriched) were cultured with various concentrations of resiquimod or imiquimod. After 24 h in culture, cell-free supernatants were collected and IFN-α was analyzed by ELISA (Gibson et al. 2002).

Suspensions containing RNA-LL37 or supernatants of dying cells were added to pDC and mDC cultures. After overnight culture, supernatants of pDCs and mDCs were collected and IFN-α, TNF-α and IL-6 were measured by ELISA (Ganguly et al. 2009). pDCs and mDCs were also stained with fluorochrome-labeled anti-CD80, anti-CD86, and anti-CD83 antibodies and analyzed by flow cytometry. mDCs were also cultured with supernatants of pDCs stimulated for 24 h with self-DNF-LL37 (Ganguly et al. 2009).

In time kinetic studies, moDCs were stimulated with R848 in the absence or presence of MDP and iE-DAP which are ligands of NOD1/2, for 30 min, 2 h, 8 h or 24 h and mRNA levels of IL-23 as well as the cumulative cytokine release were measured by qRT-PCR and sandwich-ELISA, respectively. At the mRNA level, synergistic effects of both NOD ligands with R848 are already detectable after 8 h of stimulation. In agreement with IL-23 mRNA expression, synergistic effects are detectable by ELISA after 8 h; nevertheless, these effects are even more pronounced after 24 h of stimulation (Schwarz et al. 2013).

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Relationship: 2018: Overproduction of IL-23 leads to Overproduction of IL-17 (https://aopwiki.org/relationships/2018)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease (https://aopwiki.org/aops/313)	**adjacent**	**High**	**High**

Evidence Supporting Applicability of this Relationship

In mice, application of IL-23 causes psoriatic-like epidermal hyperplasia, but this effect does not occur in IL-17A and IL-22KO mice. Therefore, it is thought that IL-17A and IL-22 play an important role downstream of IL-23 Rizzo HL. Et al. 2011 .

Recombinant mIL-23 (rmIL-23) injected into the ear of WT mice induced IL-17A and IL-22 expression, and showed ear swelling and epidermal hyperplasia. When rmIL-23 was injected into IL-22 KO mice, IL-22 was induced, but ear swelling and epidermal hyperplasia were less than in WT mice. When rmIL-23 was injected into IL-17A KO mice, IL-22 was induced, but ear swelling and epidermal hyperplasia hardly occurred. WT mice after administration of IL-22 or IL-17A inhibitor completely inhibited IL-23-induced epidermal hyperplasia. These results indicate that two cytokines, IL-22 and IL-17A, are downstream mediators of IL-23-induced changes in mouse skin and are required for the generation of IL-23-mediated skin lesions. (Hansel et al. 2011)

Key Event Relationship Description

IL-23 is important for differentiation and proliferation of Th17 cells. As a major source of IL-23, Tip-DC is present in the skin lesions of psoriatic patients and works to activate the Th17 pathway (Hansel et al. 2011).

Signaling through the heterodimeric IL-23 receptor (subunits of p19 and p40) of Th17 cells stimulates the production of proinflammatory

keratinocyte cytokines that mediate the psoriatic response and induces the production of IL-17. Th17 cells are increased in the peripheral blood and lesion skin of psoriatic patients, and IL-17 and IL-22 produced from Th17 act on epidermal keratinocytes to cause inflammatory chemokines and hyperproliferation (Michelle A. et al. 2005).

IL-17A, which is highly expressed by Th17 cells, has a direct effect on the regulation of genes expressed by keratinocytes that are involved in innate immune defense, including defensins,8, 9 S100 family proteins, lipocalin, and LL37/cathelicidin, as well as a range of CXCL chemokines that regulate neutrophil trafficking (Gilliet et al. 2004). IL-22, which is expressed by Th22 and Th17 cells, and related IL-20 family members promote keratinocyte hyperproliferation and abnormal differentiation (Krueger et al. 2012).

Evidence Supporting this KER

Biological Plausibility

IL-17A, which is highly expressed by TH17 cells, has a direct effect on the regulation of genes expressed by keratinocytes that are involved in innate immune defense, thorough expressions of defensins,8, 9, S100 family proteins, lipocalin and LL37/cathelicidin, as well as a range of CXCL chemokines that regulate neutrophil trafficking. IL-22, which is expressed by TH22 and TH17 cells, and related IL-20 family members promote keratinocyte hyperproliferation and abnormal differentiation Gilliet et al.2012 .

In vitro Reconstituted Human Epidermis (RHE) model stimulated for 48 hours with medium containing IL-17, IL-22 and TNFα mix (concentration 3 ng / mL) as psoriasis-specific cytokines. Controls were cultured in normal medium. After fixing RHE and embedding in paraffin, 4 µm sections were stained with hematoxylin-eosin or immunolabeled with anti-filaggrin, anti-S100A7, anti-hBD-2 mAb.

RHE stimulated with cytokine mix showed dramatic expression of these protein. In the RHE with normal medium, antibacterial peptide S100A7 was expressed locally, but BD-2 protein was not detected. This is due to the synergistic effect of IL-17 added to the IL-22 / TNFα combination. Filaggrin, S100A7 and BD-2 mRNA expression by RT-qPCR analysis increased 20-fold (S100A7) or -50-fold (BD-2) compared to controls. This is a downstream event that can be modeled using keratinocytes and cytokines and relies on upstream mechanisms of recruitment and activation of other innate adaptive immune cells. Bernard et al. 2012. .

Quantitative Understanding of the Linkage

Response-response relationship

KE1:

IL-23, which maintains Th17 cells, is released from TNF-a and inducible nitric oxide synthase (iNOS) -producing dendritic cells (TIP-DC). TIP-DC activates IL-17, IL-22, IL-23, and TNF-a mRNA expression in psoriatic skin. Cytokine staining analysis of peripheral blood mononuclear cell (PBMC) in patients with psoriasis showed a three-fold increase in Th17 cells compared to normal PBMC. Thl7 cells produce IL-22 and stimulate keratinocyte proliferation. IL-22 activates STAT3 and induces the production of cytokine (such as IL-8), chemokines and the synthesis of antimicrobial peptides (Zaba et al. 2005).

KE 2

The epidermis of psoriasis patients did not have many T cells, but the analysis was similar to peripheral blood and dermis. The proportion of Th17 cells in the dermis was significantly higher than that in normal skin, and TNF and IFN-g were produced from Th17 cells. Skin and peripheral blood contained a subset of Th17 cells producing IFN-g / TNF.

Keratin 16, IL-17, IFN-g, and IL-22 mRNA expression increased in psoriatic skin, but cyclosporine therapy returned these mRNA to normal levels. The average expression of IL-17 / human acidic ribosomal protein (hARP) in non-lesional skin was 0.4 compared to 10.8 in lesional skin, and cyclosporine administration returned to non-lesional levels. That IL-17 mRNA return to baseline, effective treatment supports that Th17 in psoriasis is a central pathogenic.(Lowes et al.2008)

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Relationship: 2019: Overproduction of IL-17 leads to Skin disease (https://aopwiki.org/relationships/2019)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease (https://aopwiki.org/aops/313)	**adjacent**	**High**	**High**

Evidence Supporting Applicability of this Relationship

Initiation of plaque formation in the Aldara psoriasis mouse model is dependent on RORγt +, skin infiltrating γδ T cells, and innate lymphocyte cells (ILC). Vγ4 + γδT cells and innate lymphoid cells (ILC) are the dominant and important sources of IL-17A, IL-17F, and IL-22 in the formation of acute psoriatic lesions, rather than Th cells (Pantelyushin et al. 2012).

Amyloid A: SAA, an inflammatory marker, is high in the serum of patients with psoriasis. When C57B6 mice were given SAA protein subcutaneously on the back, epidermal thickening and inflammatory cell wetting were frequent on days 5-7. Skin inflammation was significantly suppressed when IL-12 / IL-23p40 protein, a target molecule of psoriasis biologics, was administered in advance. By SAA administration, a similar reaction to psoriatic eruption was formed in the immunological reaction, and a psoriatic eruption model mouse was established. (J Dermatolog Trest. 2013; 24 (6): 477-80)

Key Event Relationship Description

Th17 cells produce the cytokines IL-17 and IL-22. IL-17 is inflammatory, promotes the migration of neutrophils to psoriatic lesions, contributes to the formation of Munro's micro-abscess, and through DCL and memory T cells, including Th17 cells and CCR6, via CCL20 Incorporate into the affected area. IL-22 causes abnormal keratinocyte proliferation by down-regulating genes that control terminal differentiation, leading to altered differentiation and keratinization. Both IL-17 and IL-22 induce keratinocyte expression of the antibacterial S100A7 (psoriacin). Nograles et al. 2008

STAT3 is important for Th17 differentiation. Cytokine signaling SOCS3-deficient mice show increased IL-17 expression by increasing STAT3 activity in response to IL-23 binding to IL-17. Associated with increased activity of STAT3 in response to IL-23 capable of binding to IL-17 and IL-17F promoters. STAT3 overexpression promotes Th17 differentiation, whereas STAT3 deficiency inhibits Th17 differentiation. STAT3 signaling from IL-6, IL-21, IL-23 regulates the expression of lineage specific master transcription factors RORγt22, 63, 66 and RORα67. It has been found that patients with psoriasis with mutations in STAT3 cannot generate a Th17 response. Martinez et al. 2008

Evidence Supporting this KER

Biological Plausibility

The biological activity of the combination of cytokines was investigated. The combination of IL-17A and IFN-γ or IL-17A and TNF-α has a synergistic effect on CXCL8 production by keratinocytes. IL-17A and IL-22 exert a synergistic effect in upregulation of β-defensin 2: BD-2 and S100A9 production] IL-1α, IL-17, IL-22, Oncostatin M: OSM, and TNFα binding are associated with increased expression of inflammatory molecules such as soriacin / S100A7 or BD-2, IL-8 in vitro by NHEK Although very potent synergistic, removal of IL-22 from the cytokine mixture reduces CXCL8 and BD-2 expression by 30% and removal of IL-17 reduces it by 70%.

Ex vivo studies on human skin explants showed upregulation of BD-2, S100A7, and CXCL8 expression in response to the same combination of cytokines, and intradermal injection of cytokines into mice resulted in neutrophil infiltration and early epidermis CXCL1, CXCL2, CXCL3, S100A9, and BD-3 expression related to epidermal thickening was increased. (Bernard et aaal. 2012)

Empirical Evidence

Resident memory tissue T cells (TRM cells) confer both resistance and immunity depending on the local microenvironment, and CD8 TRM can be tracked by phenotypic markers CD49a and CD103. Circulating effector T cells infiltrate the site of skin inflammation and turn into long-lived epidermal TRM cells when the skin inflammation is resolved. Epidermal TRM cells are thought to form pathological site-specific disease memory at the site of recurrent psoriasis.(Cheuk et al. 2014)

Single cell suspensions of epidermis and dermis were analyzed by flow cytometry within 30 hours of sampling. In active psoriasis, CD8 T cells increased about 100-fold in the epidermis compared to normal skin, whereas CD4 T cells increased 10-fold in the dermis. In healthy skin, 20-30% of epidermal CD8 T cells co-expressed integrin CD103 and CD49a, which are phenotypic markers of TRM cells. In active psoriasis, approximately half of epidermal CD8 T cells co-expressed these TRM phenotypic markers, a 100-fold increase compared to healthy skin. Cheuk et al. 2014

Uncertainties and Inconsistencies

Cytokines cannot be specified for genes associated with abnormalities in psoriatic skin. Many genes that are up-regulated in psoriatic lesions can be attributed to IFN-γ, including IL-17 and IL-22. Cytokines synthesized by Th1 / Th17 cells regulate different gene expression pathways in epidermal keratinocytes and other skin resident cells. The psoriatic transcriptome may result from activation of multiple independent pathways. Nograles et al. 2008

After daily topical application of Aldara containing imiquimod (IMQ) to humans, significant skin thickening, redness and scaling were observed 3 days later (doi: 10.1172 / JCI61862DS1). The clinical course of plaque formation on the ear and back skin and histopathology were similar. Aldara treatment resulted in impaired keratinocyte hyperproliferation and epidermal differentiation, as indicated by epidermal thickening and hyperkeratosis. There was a terminal neutrophil accumulation in the stratum corneum reminiscent of a Munro micro-abscess in psoriasis. Extensive leukocyte infiltration was observed in the dermis.(Pantelyushin et al. 2012)

Quantitative Understanding of the Linkage

Response-response relationship

KE2

High levels of Th17 cytokines were observed in psoriatic skin induced by CD4 + T cells. IL-23 p40 subunit or IL-22 significantly prevented the development of skin lesions.

IL-22-induced acantosis and inflammation were reduced in IL-22-deficient mice compared to WT mice. Blocking IL-22 increases IL-1α, IL-1β, IL-6, IL-17, IL-17F, and TNF-α. (K. A. et al. 2013)

AO

Anti-IL-17 antibody administration results in decreased keratinocyte proliferation and differentiation, leukocyte infiltration, and keratinocyte release of inflammatory cytokines. In psoriatic lesioned keratinocytes, changes in mRNA and protein expression of IL-17 regulatory products occurred. Within 2 weeks of antibody administration, the expression of LL37 (cathelicidin), β-defensin 2, S100A7, and S100A8 proteins was markedly decreased in keratinocytes, and the expression reached normal levels after 6 weeks.(Krueger et al. 2012)

Time-scale

Epidermal keratinocyte expression genes that were elevated in psoriatic lesions of patients with psoriasis with stage-type skin eruption: mRNA expression level of keratin6a and 16, s100A7A, S100A12, DEFB4, IL-1F6, CCL20, IL-17C, etc. was rapidly reduced by 700 single intravenous dose of brodalumab and decreased to non-lesional skin level 2 weeks after administration. On the other hand, leukocyte expression genes with increased expression in psoriatic lesion skin: IL-17A, IL-17F, IL-23F, IL-12B, IL-22, IFN-γ and other mRNA expression levels decreased with brodalumab administration. However, at 2 weeks after administration, the level did not decrease to the level of the non-lesional skin. Since the expression of pathophysiology-related genes is reduced prior to the decrease in the expression of leukocyte expression genes is reduced prior to the decrease in the expression of leukocyte expression genes and the decrease in the PASI score, Brodalumab is reduced expression of pathophysiology-related genes by blocking IL-17 signaling in the epidermal keratinocytes of psoriatic lesions It is possible to improve the skin eruption promptly. (Kyowa Hakko Kirin Co., Ltd.)

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AOP ID and Title:

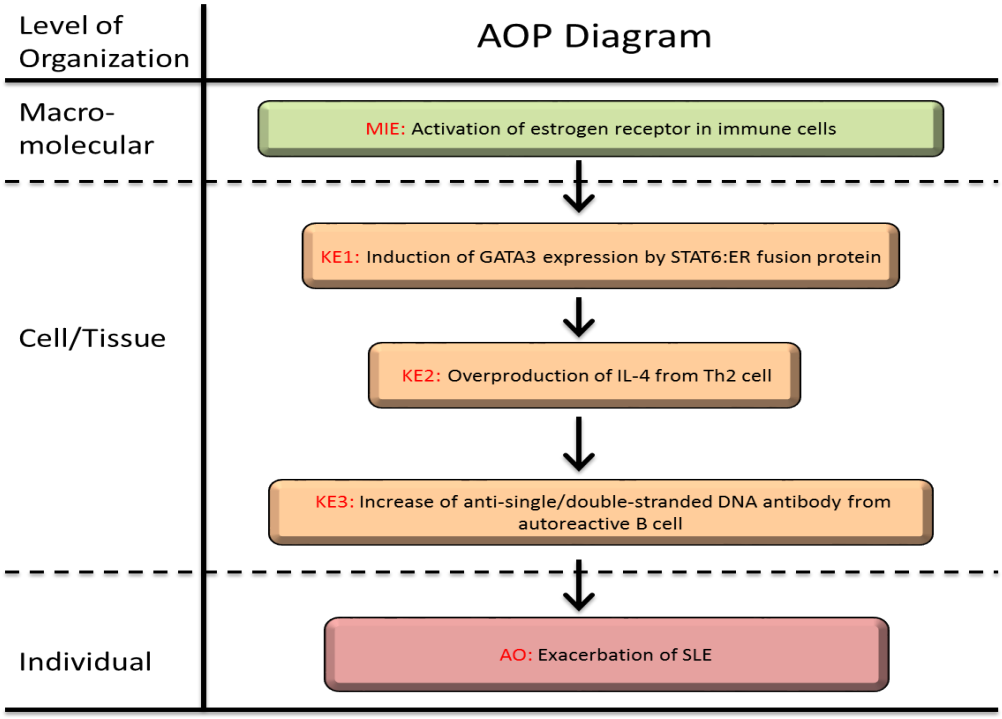
SNAPSHOT

Created at: 2020-05-18 09:23

AOP 314: Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus

Short Title: Exacerbation of SLE by activation of estrogen receptor

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.73	Included in OECD Work Plan

Abstract

This AOP describes the linkage between the activation of estrogen receptor (ER)α and the exacerbation of the autoimmune disease systemic lupus erythematosus (SLE). SLE is an autoimmune disease characterized by overproduction of a variety of anti-cell nuclear and other pathogenic autoantibodies. It is characterized by B-cell hyperactivity, polyclonal hypergammaglobulinemia, and immune complex deposition.

Estrogen Receptors (ERs), ERα and ERβ, are a group of proteins that are activated by the steroid hormone estrogen and are widely expressed in most tissue types, including most immune cells. ERs can be activated with exogenous and endogenous estrogens. Also, there are numerous xenoestrogens that exist in the environment and imitate estrogen. Bisphenol A is an example of a xenoestrogen that is considered an endocrine disrupting compound (EDC).

Estrogen Receptors (ERs), ERα and ERβ, are a group of proteins that are activated by the steroid hormone estrogen and are widely expressed in most tissue types, including most immune cells. ERs can be activated with exogenous and endogenous estrogens. Also, there are numerous xenoestrogens that exist in the environment and imitate estrogen. Bisphenol A is an example of a xenoestrogen that is considered an endocrine disrupting compound (EDC).

Binding of ER in immune cells by a xenoestrogen or endogenous ER marks the molecular initiating event (MIE), which results in induction of GATA3 expression (KE1).

One theory of immune regulation involves homeostasis between T-helper 1 (Th1) and T-helper2 (Th2) activity. Hyperactivation of ERα skew the immune system from a T helper 1 (Th1) to a Th2 profile and exacerbates autoimmune diseases and allergic diseases.

Complexes formed by the binding of ERα to stressors such as estrogen or EDC transport into cell nuclei, where they activate the transcription of specific genes. Excessive ERα-activation promotes the differentiation of naive CD4+ T cells into mature Th2 cells. This pathway leads to the overproduction of the cytokine interleukin-4 (IL-4) from Th2 cells and anti-single/double-stranded DNA antibody from autoreactive B cell are increased, which results in the adverse outcome of exacerbated SLE.

We have identified a number of key events along this pathway and determined the key event relationships, based on which we have created an AOP for activation of ERα in immune cells leading to exacerbated SLE.

Background

It has long been appreciated that most autoimmune disorders are characterized by increased prevalence in females, suggesting a potential role for sex hormones (estrogen) in the etiology of autoimmunity. ERs are involved in a wide range of physiological function. Women generally exhibit a stronger response to a variety of antigens including ER ligands than men, which is perhaps one reason that they are more prone to develop autoimmune and allergic diseases such as SLE in greater severity than men. This AOP could be helpful to assess the type of Th2 dominant autoimmune disorders

Humans and mammals have two ligand-activated transcription factors that bind estrogen, encoded by separate genes, estrogen receptor alpha (ESR1/ERα) and estrogen receptor beta (ESR2/ERβ) (Maria, B. 2015). The estrogen receptors are composed of several domains important for hormone binding, DNA binding, dimer formation, and activation of transcription (Green S. 1986, Kumar V. 1986, Warnmark A. 2003). The ERs' expression patterns and functions vary in a receptor subtype, cell- and tissue-specific manner. In the adult human, large-scale sequencing approaches show that ERα mRNA is detected in numerous human tissues, with the highest levels in the uterus, liver, ovary, muscle, mammary gland, pituitary gland, adrenal gland, spleen and heart, and at lower levels in the prostate, testis, adipose tissue, thyroid gland, lymph nodes and spleen (Fagerberg L. 2014, Sayers EW. 2012) (www.ncbi.nlm.nih.gov/UniGene). In the same data sets, human ERβ mRNA is primarily detected in the lung and testis. There is increased ERα and decreased ERβ mRNA expression in PBMCs of SLE patients (Inui A. 2007). Although ERs are widely expressed in most tissue types, including most immune cells, this AOP mainly addresses hyperactivation of ERα in immune cells.

The effects of ERα signaling on T cells appear to be estrogen-dose dependent, i.e., low doses of estrogen stimulate a Th1 response, but higher doses promote a Th2 response (Priyanka HP. 2013). This AOP describes events occurring when high levels of estrogen shift the Th1/Th2 balance toward increased Th2 activity

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	1710	Activation of estrogen receptor in immune cells (https://aopwiki.org/events/1710)	Activation of estrogen receptor
	KE	1711	Induction of GATA3 expression by STAT6:ER fusion protein (https://aopwiki.org/events/1711)	Induction of GATA3 expression
	KE	1712	Overproduction of IL-4 from Th2 cell (https://aopwiki.org/events/1712)	Overproduction of IL-4
	KE	1713	Increase of anti-single/double-stranded DNA antibody from autoreactive B cell (https://aopwiki.org/events/1713)	Increase of autoantibody production

Sequence	Type	Event ID	Title	Short name
	AO	1714	Exacerbation of systemic lupus erythematosus (https://aopwiki.org/events/1714)	Exacerbation of SLE

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Activation of estrogen receptor in immune cells (https://aopwiki.org/relationships/2020)	adjacent	Induction of GATA3 expression by STAT6:ER fusion protein	Moderate	Moderate
Induction of GATA3 expression by STAT6:ER fusion protein (https://aopwiki.org/relationships/2021)	adjacent	Overproduction of IL-4 from Th2 cell	Moderate	Moderate
Overproduction of IL-4 from Th2 cell (https://aopwiki.org/relationships/2022)	adjacent	Increase of anti-single/double-stranded DNA antibody from autoreactive B cell	Moderate	Moderate
Increase of anti-single/double-stranded DNA antibody from autoreactive B cell (https://aopwiki.org/relationships/2023)	adjacent	Exacerbation of systemic lupus erythematosus	Moderate	Moderate

Stressors

Name	Evidence
Estrogen	High
Bisphenol A	Moderate

Overall Assessment of the AOP

The immune system is the most complex and sophisticated in the body's defense mechanisms . Estrogen plays a role in controlling the immune balance. Hyperactivation of ERα can skew the immune system from a Th1 to a Th2 profile. This Th1/Th2 shift is one of the most important immunologic changes during gestation and occurs due to a progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress Th1-mediated responses and stimulate Th2-mediated responses (Doria A. 2006). Incidence of flare in patients with SLE is increased during pregnancy and within the 3-months postpartum (Amanda E. 2018). Thus, ERα activation can potentially induce immunoactivation-derived adverse outcomes, one effect of which could be exacerbation of SLE. The present AOP focused on ERα activation-induced exacerbation of SLE.

In general, ERα is activated when bound to a stressor, which subsequently binds to estrogen response elements (EREs) to transactivate or to suppress specific target genes. In naive CD4+ T cells, T cell expansion shifts toward a Th2 phenotype that produces Th2 cytokines such as IL-4, IL-5, IL-10, and IL-13, thereby increasing antibody production from autoantibody-producing B cells. We have identified a number of key events (KE) along this pathway and used these key event relationships (KER) to create an AOP that describes the activation of ERα leading to exacerbation of SLE.

Ordinary estrogen levels in women are 20-30 pg/mL during diestrus, 100-200 pg/mL during estrus, and 5000-10000 pg/mL during pregnancy (Offner H. 2000). While BPA binds in some assays with less than 2000-fold affinity compared to the binding of estradiol to estrogen receptors, it still has dramatic effects (Krishnan AV. 1993). Since each KE is quantifiable and shows similar dose responses with the stressors in vitro, the activation of ER leading to exacerbation of SLE comprise a suitable AOP. Additionally, each KER is based on sufficient scientific evidence and exhibits no contradiction with dose response of adjacent KE.

Since ERα expresses in the cells of a vast variety of (vertebrate) species (Maria B. 2015) and there is common functionality in the immune systems of at least humans and mice, this AOP might be applicable to many mammal species, including humans and rodents.

Essentiality of KEs – what would be good is to have a table listing references that have demonstrated occurrence of individual KEs and their relationship with the AO.

Evidence assessment – here listing knockout or overexpression studies that intervene with a KE to show its essentiality to the AO

Quantitative assessment – if you have this informaiton

[Otsubo2]We will reconsider it and revise later.

[SH3]It seems like KE1 is not needed as it is not described much.

[Otsubo4]We want to discuss about it in WebEX meeting.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Sex Applicability

Sex	Evidence
Mixed	High

The proposed AOP describes the activation of ERα leading to exacerbation of SLE is dependent on estrogen level, which means it varies with life stage, sex, and age. SLE frequently develops or progresses when sympathoadrenomedullary and gonadal hormone levels are altered during pregnancy, the postpartum period, or menopause as well as during exposure to estrogen and includes the risk of preeclampsia or premature birth (Wilder RL. 1999, Whitelaw DA. 2008). Women using oral contraceptives that contain estrogen or undergoing hormone replacement therapy are susceptible to major flare ups and exacerbation of the disease (Whitelaw DA. 2007).

Since stressor-induced outcomes in humans are mimicked by similar responses in rodents, Th2 dominant conditions induced by activation of ERα is considered likely to occur in a variety of mammalian species.

Essentiality of the Key Events

Stressor , MIE and later events: ER knock out (KO) mice

It has been determined in a murine model of SLE that ERα is required for disease progression and that ERα deficiency impedes the course of the disease (Bynote KK. 2008).

The NZB/W F1 mouse is the oldest classical model of lupus generated by the F1 hybrid between the NZB and NZW strains. Both NZB and NZW display limited autoimmunity, while NZB/W F1 hybrids develop severe lupus-like phenotypes comparable to that of lupus patients. SLE in the NZB/W F1 strain is strongly biased toward females, and this is at least in part due to estrogen levels. Indeed, ovariectomy of NZB/W F1 mice not only delayed onset of the disease but also decreased autoantibody titer. Meanwhile, restoration of estradiol in ovariectomized NZB/W F1 mice reestablished high numbers of autoantibody-producing (DNA-specific) B cellsDNA-specific B cells, and thereby suggests a pathogenic role of estrogen in lupus (Daniel P. 2011).

In females of the lupus-prone NZB/NZW F1 strain, disruption of estrogen receptor-α (ERα or Esr1) both attenuated glomerulonephritis and increased survival. ERα deficiency also retarded development of anti-histone/DNA antibodies, suggesting that ERα promotes loss of immunologic tolerance. The presence of many autoantibodies is a hallmark of SLE. In particular, autoantibodies directed to double-stranded DNA (dsDNA) are characteristic (Isenberg DA. 2007). ERα deficiency in NZB/NZW F1 males increased survival and reduced anti-dsDNA antibodies, suggesting that ERα also modulates lupus in males (Bynote KK. 2008).

KE1 and later events: Stat6 KO mice

CD4 T cells from Stat6-knockout mice are not able to drive Th2 differentiation and cell expansion under null Th cell (ThN) conditions with added with IL-4 (Zhu J. 2001)

KE1 and later events: GATA3 KO mice

Th2 differentiation is completely abolished both in vitro and in vivo when GATA3 is conditionally deleted in peripheral CD4 T cells. Th2 cells from both knockout animals showed reduction in IL-4, IL-5, IL-13, and IL-10 production. Conversely, IFN-γ production was increased even under Th2 conditions (Zhu J. 2004, Pai SY. 2004).

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Appendix 1

List of MIEs in this AOP

Event: 1710: Activation of estrogen receptor in immune cells (https://aopwiki.org/events/1710)

Short Name: Activation of estrogen receptor

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	MolecularInitiatingEvent

Stressors

Name
Estrogen
Bisphenol A

Biological Context

Level of Biological Organization
Molecular

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

ERα is mainly expressed in uterus, prostate (stroma), ovary (theca cells), testes (Leydig cells), epididymis, bone, breast, various regions of the brain, liver, and white adipose tissue (Dahlman-Wright K. 2006). ERs is widely expressed in most tissue types including most immune cells (Couse JF. 1997). ERα and ERβ show a high degree of similarity when compered at the amino acid level (Dahlman-Wright K. 2006). Interspecies sequence identities for the entire ERα receptor are 88.5% (human-mouse), 87.5% (human-rat), and 97.5% (mouse-rat). For the ligand binding domain (ERα-LBD) alone, the interspecies sequence identities are 95.5% (human-mouse), 95.1% (human-rat), and 99.2% (mouse-rat) (White R. 1987). ERα is found in female reproductive organs, yet is robustly expressed in kidney, liver, heart, and lungs in males and females, as well as on most immune cells (Chelsea C. 2017).

Key Event Description

Estrogen receptor alpha (ERα) was discovered in the late 1960s and was cloned and characterized in 1985 (Melissa C. 2011). ERα and ERβ show a high degree of similarity when compared at the amino acid level (Dahlman-Wright K. 2006). 17β-estradiol (E2) activates ERα and ERβ with the same affinity although they share only 56% similarity in their ligand binding domains (Monroe DG. 2005, Papoutsi Z. 2009). The hormone binding domain of the estrogen receptor is required not only for binding estradiol but also to form stable homodimers of the protein and mediate transcriptional activation by the receptor. A direct genomic interaction occurs between the estrogen receptor (ER) ligand complex and specific sequences of DNA known as estrogen response elements (ERE). (Parker MG. 1993, Goldstein RA. 1993, Sasson S. 1991, Brandt ME. 1997). Transcriptional activation by ERα is mediated by two distinct activation functions: the constitutively active AF-1 domain, located in the N-terminal domain of the receptor protein, and the ligand-dependent AF-2 domain, located in the C-terminal domain of the receptor protein (Delaunay F. 2000). In addition to above classical mechanism, ERα is also able to play roles both in ER binding and transcriptional activation; phosphorylation of ER and other proteins involved in transcriptional activation with cellular amounts of coactivators and adaptor proteins (Carolyn MK. 2001).

ERs are expressed in a variety of immunocompetent cells, including CD4+ (Th1, Th2, Th17, and Tregs) and CD8+ cells and macrophages (Salem ML. 2004, Robinson DP. 2014). One recent study examined ERα expression in resting and activated PBMC subsets and found that ERα was expressed at higher levels in CD4+ T cells than B cells (Melissa C. 2011).

How it is Measured or Detected

Recombinant human estrogen receptor hormone-binding domain (HBD) fragment is isolated from Escherichia coli. Purified HBD peptide is assayed for their ability to bind estradiol, [3H] estradiol binding using low concentrations (0.15 nM), by Radioreceptor Assay. Moreover HBD dimer dissociation is measured using size exclusion chromatography (Brandt ME. 1997).

On the other hand, a conditionally active form of STAT (the signal transducers and activator of transcription) 6 by fusing the HBD of a modified form of the mouse estrogen receptor (ER) gene is prepared as STAT6-ER fusion protein (STAT6:ER). 4-Hydroxytamoxifen (4-HT), estrogen analogue, (Research Biochemicals Institute, Natick, MA) was used to activate STAT6 fusion protein. M12.4.1 cells, transfected with the luciferase reporter gene by inserting three copies of human STAT6 binding site oligonucleotide, are used nuclear extracts and electrophoretic mobility shift assay (EMSA) with 1 μM 4HT. STAT6:ER DNA-binding activity is strongly and rapidly (within 1 hr) induced after addition of 4HT to these cells. BA/F3 cells prepared as the same manner are stimulated with 1 μM 4HT for 24 h at 37°C. The cells were harvested and assayed for luciferase activities using a Luciferase Assay Kit (Promega, Madison, WI). (Kamogawa et al. 1998).

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List of Key Events in the AOP

Event: 1711: Induction of GATA3 expression by STAT6:ER fusion protein (https://aopwiki.org/events/1711)

Short Name: Induction of GATA3 expression

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Organ term

Organ term
immune system

Domain of Applicability

Involvement of GATA3 and STAT6 in Th2 cell development through ER is common in humans, rodents, and other mammalian species (Ho IC. 2009). A constitutively activated form of Stat6 introduced into CD4 T cells resulted in both Th2 differentiation and enhanced cell expansion. Stat6 is not only necessary but also sufficient to drive IL-4-mediated Th2 differentiation and cell expansion in naive CD4 T cells (Zhu J. 2001). CD4 T cells from Stat6-knockout mice are not able to drive Th2 differentiation and cell expansion under ThN conditions with added with IL-4 (Zhu J. 2001).

Key Event Description

Transcription factors are critical for Th cell differentiation and cytokine production. Cell fate determination in each lineage requires at least two types of transcription factors: the master regulators as well as the signal transducers and activator of transcription (STAT) proteins (Zhu J. 2010). The ability of STAT6: ER to induce a Th2 phenotype correlates with the induction of GATA-3 mRNA expression. GATA3 is the Th2 master regulator (Zhu J 2010, Sung-Yun. 2004, Zhu J. 2004, Zheng W. 1997, Zhang DH. 1997), but it also plays important roles in multiple steps of CD4 T cell development (Ho IC. 2009).

How it is Measured or Detected

Purified naive T cells were cultured and expanded under Th1 culture conditions in the presence or absence of 0.3 μM 4-HT (Research Biochemicals Institute) for 2 weeks starting from days 1, 7, 14, or 21. GATA-3 mRNAs can be measured using RNase protection assay in developing Th1 cells. RNase protection assay was performed with RiboQuant multiprobe kit (PharMingen) following the manufacturer's method using GATA-3. Stat6:ER Th1 cells expressed significant amounts of both GATA-3 mRNAs in a 4-HT-dependent manner. (Kurata H. 1999, Zhu J. 2001).

Constitutively activated Stat6 (Stat6VT) is primed under null Th cell (ThN) conditions in the absence of human (h)IL-4. The expression level of Gata3 in this primed cells are checked by RT-PCR (Zhu J. 2001).

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Event: 1712: Overproduction of IL-4 from Th2 cell (https://aopwiki.org/events/1712)

Short Name: Overproduction of IL-4

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
T-helper 2 cell

Organ term

Organ term
immune system

Key Event Description

Th2 cells produce IL-4, which stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. The receptor for IL-4 is IL-4Rα, which expresses in B cells. IL4 also plays an important role in the development of certain immune disorders, particularly allergies and some autoimmune diseases and especially when there is Th2 polarization.

How it is Measured or Detected

Purified naive T cells were activated and infected with RV-Stat6:ER. The cells were cultured and expanded under Th1 culture conditions in the presence or absence of 0.3 μM 4-HT (Research Biochemicals Institute) for 2 weeks starting from days 1, 7, 14, or 21 and the cells were analyzed for cytokine (IL-4) expression by flow cytometer analysis of intracellular cytokine production or cytokine ELISA (Kurata H. 1999, Zhu J. 2001).

Single-cell suspensions of lymph nodes removed from BALB/c mice 7 days after priming with KLH absorbed to aluminium hydroxide adjuvant in the footpads, were prepared and cultured in vitro with KLH in the absence or presence of either BPA (0.1, 1, 10, 50 and/or 100 μM) or NP. After 4 days, the levels of IL-4 and IFN-γ in the cell supernatants were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) and mRNA levels of IL-4, IL-6 and IL-10 in the cells were assayed by reverse transcription–polymerase chain reaction (RT–PCR) (Lee MH. 2003). To evaluate the effects of exposure to BPA in adulthood, male Leishmania major– susceptible BALB/c and –resistant C57BL/6 mice were subcutaneously injected with BPA (0.625, 1.25, 2.5 and 5 μmol) dissolved in corn oil 1 week before being infected with L. major. A single cell suspension containing splenocytes from each mouse was incubated in 24-well tissue-culture plates in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 IU/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Cells were stimulated with L. major antigen (3 μg/mL) during the cultivation. Culture supernatants were collected 48 hr later. Concentrations of IL-4, IL-10, IL-13, and IFN-γ in culture supernatants were determined using CBA kits (Huimin Y. 2008).

Th2 cell-related cytokine (IL-4 and -10) in BPA (50 μM)-stimulated primary cultured mouse lymphocytes were evaluated using immunoblot analysis and reverse-transcription polymerase chain reaction (RT-PCR) (Lee et al. 2010).

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Event: 1713: Increase of anti-single/double-stranded DNA antibody from autoreactive B cell (https://aopwiki.org/events/1713)

Short Name: Increase of autoantibody production

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
B cell

Organ term

Organ term
immune system

Key Event Description

In the development of T-cell dependent antibody producing cells, the interaction between IL-4 and its receptor delivers the first signal for switching to IgE. IL-4 produced by Th2 stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. The engagement of CD40 on B cells by CD154 (CD40L) expressed on T cells and DC provides the second signal required for switching to IgE.

In a study to investigate a novel subpopulation of B-1 cells and its roles in murine lupus, anti-double-stranded DNA (anti-dsDNA) autoantibodies were preferentially secreted by a subpopulation of CD5+ B-1 cells that expressed programmed death ligand 2 (L2pB1 cells) (Xuemei et al. 2009). A substantial proportion of hybridoma clones generated from L2pB1 cells reacted to dsDNA. L2pB1 cells are potent antigen-presenting cells and a dramatic increase of circulating L2pB1 cells in lupus-prone BXSB mice correlates with elevated serum titers of anti-dsDNA antibodies (Xuemei et al. 2009).

Bisphenol-A (BPA) as well as E2 and DES enhanced anti-Br-RBC autoantibody production by B1 cells in vivo. IgM production by B1 cells in the presence of EDs was more prominent on aged BWF1 mice developing lupus nephritis. B1 cells from aged mice exhibited increased expression of ERα mRNA compared to young mice (Yurino H. 2004).

How it is Measured or Detected

For the detection of anti-DNA antibodies in serum of female NZB/W F1 mice administrated of the estrogen antagonist tamoxifen, enzyme-linked immunosorbent assay (ELISA) was carried out. For the quantitated of total B cells and CD5+B cells expression in spleen and in peritoneal exudates were analyzed with fluorescence activated cell sorting (FACScan) (Wu et al. 2000). For the B cell subset analysis (including immature (transitional T1 and T2) and mature (MZ and follicular)) in BALB/c R4Ag-gamma 2b transgenic mice administrated the tamoxifen were performed with FACScan (Peeva et al. 2005).

In another study, used ERα deficiency in NZB/W F1 mice, autoantibody (anti-dsDNA antibodies) development and concentration was assessed by ELISA using serum isolated from blood collected monthlyvia (Bynote et al. 2008).

Using female NZB/WF1 mice, silastic implants containing the powdered form of endocrine disruptors were placed subcutaneously on the back of ovariectomized mice, and 3 to 4 months blood samples were collected peritoneal. 4 months after implantation, peritoneal lavage cells and splenic cells were obtained from mice. Anti-DNA antibody was measured in ELISA using ssDNA for the culture supernatant of and dsDNA for the serum. To examine the effect of EDs on autoantibody production by B1 cells, a PFC assay using autologous bromelain-treated erythrocytes (Br-RBC) was conducted. To evaluate autoantibody (IgG) production including plaque forming cell (PFC) assay for anti-RBC Ab. It has been reported

that B1 cells produce autoantibody against phosphatidylcholine expressed on bromelain-treated red blood cells (Br-RBC) using PFC assay (Yurino H. 2004).

To examine a direct effect of endocrine disruptors on IgM antibody production by B1 or B2 cells, B1 cells were prepared from peritoneal cells and B2 cells from spleen, B1 or B2 cells were cultured in the presence of endocrine disruptors (E2: 100 nM, DES: 100 nM, BPA: 1 μM) for 4 days. The amount of total IgM and IgM anti-DNA Ab in the culture supernatant was measured by ELISA. Expression level of ERα and ERβ genes in B

cells was examined by RT-PCR and quantitative real-time PCR analysis (Yurino H. 2004).

For the investigate the in vitro effects of 17β-estradiol (E2) on spontaneous immunoglobulin production by human PBMCs, PBMCs from healthy human volunteers were cultured with E2. Levels of IgG and IgM and cytokine activity were measured by ELISA. Proliferation was determined by [3H]-thymidine uptake. The cell viability was assessed by a trypan blue exclusion test (Kanda et al. 1999).

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List of Adverse Outcomes in this AOP

Event: 1714: Exacerbation of systemic lupus erythematosus (https://aopwiki.org/events/1714)

Short Name: Exacerbation of SLE

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	AdverseOutcome

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Exacerbation of SLE is common in humans and rodents, and is considered likely to occur in other animal species, as well. SLE is an autoimmune disease that occurs primarily in women (9:1 compared to men) (Rider et al., 2001). SLE is an autoimmune disease that affects predominantly women during reproductive years, and its evolution is altered by hormonal events such as menses, menopause, and especially pregnancy (Luis et al., 2014). The incidence of SLE is markedly increased in females of child-bearing age (Grainne et al., 2013). Th1/Th2 shift is one of the most important immunologic changes during gestation. It is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses. For this reason, Th1-mediated diseases, such as rheumatoid arthritis, tend to improve, while Th2-mediated diseases, such as systemic lupus erythematosus (SLE) tend to worsen during pregnancy (Doria et al., 2006).

Key Event Description

SLE is an autoimmune disease characterized by overproduction of a variety of anti-cell nuclear and other pathogenic autoantibodies. It is characterized by B-cell hyperactivity, polyclonal hypergammaglobulinemia, and immune complex deposition. Epstein– Barr virus (EBV) has been identified as a possible factor in the development of lupus. Over 100 drugs have been reported to cause drug-induced lupus (DIL), including a number of the newer biologics and antiviral agents. Although the pathogenesis of DIL is not well understood, these drugs may alter gene expression in CD4+ T cells by inhibiting DNA methylation and induce over-expression of lymphocyte function-associated antigen 1, thus promoting autoreactivity. Generally, sunlight is the most obvious environmental factor that may exacerbate SLE. High estrogen levels and BPA-induced ER activation skewed T cells toward a Th2 phenotype, thereby inducing hyperactivity by B-cells, which leads to exacerbation of SLE. T cell dysfunction is a characteristic of SLE, which is also associated with high levels of autoantibodies (Crispin et al. 2010).

How it is Measured or Detected

Most of the mouse models of lupus produce autoantibodies and develop immune complex glomerulonephritis. For the disease onset, mice can monitor by proteinuria levels, body weights, blood urea nitrogen and appearance over time. Additionally, serum levels of anti-dsDNA, anti-glomerular antigens (GA), total IgG can measure by ELISA. (Gabriela et al., 2019, Yurino et. al.,2004, John et. al.,2008, Wang et. al.1996).

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2020: Activation of estrogen receptor leads to Induction of GATA3 expression (https://aopwiki.org/relationships/2020)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	**adjacent**	**Moderate**	**Moderate**

Key Event Relationship Description

Stressors bind to the ERs in immune cells, a ligand-activated transcription factor that regulates transcription of target genes in the nucleus or located in or adjacent to the plasma membrane (Deroo BJ. 2006). ERα is a nuclear hormone transcription factor that classically binds ligand stressors estrogen or EDC, further stabilizing dimers that subsequently bind estrogen response elements to transactivate or suppress specific target genes.

Evidence Supporting this KER

Biological Plausibility

The GATA3 expression induced by TNF-α was enhanced in the presence of BPA. However, the T-bet expression did not change when tested at various culture conditions (Guo H. 2010, Uemura Y. 2008). Naive Th cells primed by BPA/TNF-α-matured DCs differentiated into Th2 cells with characteristically high IL-5/IFN-γ, IL-10/IFN-γ, and IL-13/IFN-γ ratios. However, the IFN-γ production was not affected at all, thus indicating that

Th2 bias was induced by enhanced Th2 cytokine production (Guo H. 2010, Uemura Y. 2008). Also, dendritic cells exposed to BPA (100 nM) and TNF-α produced high levels of IL-10 relative to IL-12, and this induced Th2 deviation (Liu Y. 2009).

Uncertainties and Inconsistencies
Dendritic cells exposed to human exposure-relevant concentrations of BPA (10-100 nM) preferentially skewed T cells toward a Th2 phenotype. Th cells were primed by BPA/TNF-α-DCs. The administration of 17β-estradiol enhanced the differentiation of dendritic cells and increased IFN-γ production by dendritic cells in C57BL/6 mice.

Quantitative Understanding of the Linkage

Response-response relationship
When estrogen levels are low, T cell expansion shifts toward a Th1 phenotype that produces IL-12, TNF-α, and IFN-γ. This response results in cellular immunity inducing inflammation and exacerbating cellular autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), and experimental autoimmune encephalomyelitis (EAE) rather than SLE.

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Melissa, and Gary 2011). Low serum levels (60–100 pg/mL or 0.26–0.43 nM) of estradiol have been shown to increase Th1 T-cell development in vitro through an ERα mediated mechanism (Maret et al. 2003). Treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated disease, while high doses (>1000 pg/ml or 4.3 nM), which mimic pregnancy levels, prevented EAE onset and polarized T-cells to a Th2 phenotype in the EAE model (Bebo et al. 2001). High levels of estrogen during pregnancy have been reported to ameliorate T cell mediated diseases such as multiple sclerosis (Korn-Lubetzki et al. 1984).

Known modulating factors
The Th1/Th2 shift is one of the most important immunologic changes during the menstrual cycle and gestation. Immune activity shifts across the menstrual cycle, with higher follicular-phase Th1 cell activity and higher luteal-phase Th2 cell activity (Tierney et al. 2015). This is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses (Doria et al. 2006).

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Relationship: 2021: Induction of GATA3 expression leads to Overproduction of IL-4 (https://aopwiki.org/relationships/2021)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	**adjacent**	**Moderate**	**Moderate**

Key Event Relationship Description

Th2 cells produce IL-4, IL-5, IL-10, and IL-13, meanwhile Th1 cells produce IL-12, TNF-α, and IFN-γ. During Th2 polarization, IL-4 produced by Th2 cell. IL-12 plays a central role in promoting the differentiation of naive CD4+ T cells into mature Th1 effector cells. Secretion of IL-10 from Th2 has been suggested to downregulate the DC-derived IL-12 production and lead to a Th2 differentiation (Aste-Amezaga M. 1998).

Evidence Supporting this KER

Biological Plausibility

IFN-γ is noticeably reduced in pregnant women compared with non-pregnant women or in response to high levels of estrogen (Kruse et al. 2000). Thus, pharmacological or pregnancy levels of estrogen may skew the immune system from a Th1 to a Th2 profile (Ebru et al. 2011). Th2 differentiation is completely abolished both in vitro and in vivo when GATA3 is conditionally deleted in peripheral CD4 T cells from GATA-3-deficient (FF and FF cre) mice (Sung-Yun. 2004, Zhu J. 2004). Antigen-specific immune response is evaluated with lymphocyte from FF and FF cre mice injected with KLH, and cytokine production was measured by sandwich ELISA (Sung-Yun. 2004). Mouse lymphocytes stimulated with a massive amount of BPA (50 μM) were Th2 polarized, with prominent elevation of IL-4 as well as IL-10 (Lee MH. 2010). Similarly, BPA enhanced IL-4 production in antigen-activated T cells by ELISA or RT-PCR, although the concentrations of BPA that they utilized (10–50 μM) were high (Lee MH. 2003). In this experiment, IL-4 level is confirmed baseline when treated with anti-CD4 mAb. Exposure to BPA in adulthood mice promoted antigen-stimulated levels of IL-4, IL-10, and IL-13, but not IFN-γ (Huimin et al. 2008).

Empirical Evidence

The proliferation of Stat6:ER Th1 cells was enhanced in a dose-dependent manner on days 10 and 31 after polarization by [^{3}H]thymidine incorporation (the effective concentration of 4-HT was between 0.08 and 2 μM, and the toxic concentration was greater than 5 μM) (Kurata H. 1999, Zhu J. 2001).

Uncertainties and Inconsistencies

The essential transcription factors of Th2 are GATA-3 and STAT5. Activation of GATA-3 and STAT5 induce IL-4 production in naïve CD4 T cells. IL-4-mediated STAT6 activation promotes Th2 differentiation (Kaplan MH. 1996, Shimoda K. 1996, Takeda K. 1996).

Quantitative Understanding of the Linkage

When estrogen levels are low, T cell expansion shift toward a Th1 phenotype that produces IL-12, TNF-α, and IFN-γ. This response results in cellular immunity inducing inflammation and exacerbating cellular type autoimmune disease such as multiple sclerosis (MS) and EAE rather than SLE.

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Cunningham and Gilkeson, 2011). Treatment with low serum levels (60–100 pg/mL or 0.26–0.43 nM) of estradiol increased Th1 T-cell development in vitro by acting through an ERα mediated mechanism (Maret et al. 2003). Treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated disease, while high dose levels (>1000 pg/ml or 4.3 nM), which mimic pregnancy levels, prevented EAE onset and polarized T-cells to a Th2 phenotype in the EAE. (Bebo et al. 2001). High levels of estrogen during pregnancy have been reported to ameliorate T cell mediated diseases such as multiple sclerosis (Korn-Lubetzki et al. 1984).

IL-4 may serve multiple roles in the development of lupus: it may enhance autoantibody production via its direct B-cell effects, protect against autoimmunity via its T-cell suppressor effect, or perpetuate tissue damage via its direct effects on target organs (Ram Raj Singh 2003).

Known modulating factors

The Th1/Th2 shift is one of the most important immunologic changes during gestation. This is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses (Doria et al. 2006).

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Relationship: 2022: Overproduction of IL-4 leads to Increase of autoantibody production (https://aopwiki.org/relationships/2022)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	**adjacent**	**Moderate**	**Moderate**

Key Event Relationship Description

The receptor for IL-4 is IL-4Rα, which expresses in B cells. Th2 cells secrete cytokines IL-4 that upregulate antibody formation via B cells. Naive B cells that have not yet encountered antigen express immunoglobulin M and immunoglobulin D on their surface. During an immune response, B cells can express different immunoglobulin heavy chain isotypes sharing the same variable–diversity–joining (VDJ) region. This isotype-switching recombination allows a B-cell clone to produce antibodies with the same specificity for antigens but with different effector functions. To switch to a particular isotype, a B cell needs two signals, one cytokine-dependent and the other CD40-dependent. In B cells, estrogen-mediated events could occur through the CD40/CD40L costimulatory pathway. Estrogen can also enhance differentiation of immature DCs into mature functional DCs and regulate the expression of cytokines and chemokines such as IL-6, IL-10, CXCL8, and CCL2 (Liu Y. 2009, Guo H. 2010). This increase the number of B cells producing autoantibodies.

Evidence Supporting this KER

Biological Plausibility
Lack of ERα, in either male or female mice, did not increase B cell precursors (Smithson G. 1998).

Estrogen upregulates CD40L on B and T cells from SLE patients (Desai-Mehta A. 1996, Li X. 2006), and CD40L expression on B cells is increased two-fold in SLE patients (Díaz-Alderete A. 2004). Whereas anti-CD40L antibodies downregulate CD86 expression on normal and SLE B lymphocytes, blockade of CD86 only diminishes anti-DNA antibody production by SLE B cells (Nagafuchi H. 2003). Moreover, mice overexpressing CD40L develop a lupus-like disease with high levels of antibodies to nuclear antigens, DNA, and histones, as well as glomerulonephritis (Higuchi T. 2002). It is possible that this estrogen modulated elevation in CD40/CD40L crosstalk as well as stimulation via CD86 synergizes in the exacerbation of SLE by promoting autoantibody secretion as well as activation of T cells (Karpuzoglu E. 2011). In a murine model of SLE, BPA increased the number of B cells producing autoantibodies, and IgM antibody secretion by B1 cells was augmented (Yurino et al. 2004).

Direct exposure of PBMCs from SLE patients to 17β-estradiol induces secretion of anti-dsDNA antibodies and enhances the secretion of Igs, in particular IgG (Kanda et al. 1999).

Empirical Evidence
CD23 on M12.4.1 cells, transfected with the luciferase reporter gene by inserting three copies of human STAT6 binding site oligonucleotide, is up-regulated with treatment1 μM 4HT for 16 hr (Kamogawa et al. 1998).

The production of IgA and IgG2a was increased in B cells from mice fed BPA (Goto et al. 2007). Similarly, in mice exposed prenatally to BPA and then immunized in adulthood with hen egg lysozyme (HEL), the anti-HEL IgG2a measured three weeks later was elevated (Yoshino et al. 2004). These Ig can be measured by ELISA. The administration of the estrogen antagonist tamoxifen diminishes anti-DNA antibody levels by ELISA as well as decreases percentages of total B cells and CD5+ B cells by FCM (Wu et al. 2000). Tamoxifen Blocks Estrogen-Induced B Cell Maturation but not survival (Peeva et al. 2005). ERα deficiency in (NZB×NZW) F1 female mice downregulated levels of anti-dsDNA IgG antibodies, and the absence of ERα In (NZB×NZW) F1 males resulted in decreased anti-dsDNA antibodies (Bynote et al. 2008).

Quantitative Understanding of the Linkage

Response-response relationship
When estrogen levels are low, T cell expansion shift toward a Th1 phenotype that produces IL-12, TNF-α, and IFN-γ. This response results in cellular immunity inducing inflammation and exacerbating cellular type autoimmune disease such as multiple sclerosis (MS) and EAE rather than SLE.

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Cunningham and Gilkeson, 2011). Treatment with low serum levels (60–100 pg/mL or 0.26–0.43 nM) of estradiol increased Th1 T-cell development in vitro by acting through an ERα mediated mechanism (Maret et al. 2003). Treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated disease, while high dose levels (>1000 pg/ml or 4.3 nM), which mimic pregnancy levels, prevented EAE onset and polarized T-cells to a Th2 phenotype in the EAE. (Bebo et al. 2001). High levels of estrogen during pregnancy have been reported to ameliorate T cell mediated diseases such as multiple sclerosis (Korn-Lubetzki et al. 1984).

Time-scale
The Th1/Th2 shift is one of the most important immunologic changes during gestation. This is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses (Doria et al. 2006).

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Relationship: 2023: Increase of autoantibody production leads to Exacerbation of SLE (https://aopwiki.org/relationships/2023)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	**adjacent**	**Moderate**	**Moderate**

Key Event Relationship Description

SLE patients appear to produce significant amounts of the anti-double-stranded DNA (anti-dsDNA) autoantibodies that cause the disease. Activation of autoantibody-producing B cells only serves to exacerbate that condition.

Evidence Supporting this KER

Biological Plausibility

SLE has been seen to flare up during pregnancy (Petri et al., 1991). Female MRL/lpr mice that developed lymphadenopathy and a lupus-like disease also exhibited a 50% higher mortality rate than males at 5 months of age (Carlsten H. 1992).

In (NZB×NZW) F1 mice too, females develop signs of SLE several months before males, with severe autoimmune hemolytic anemia, glomerulonephritis, and autoantibodies to single-stranded DNA, doublestranded DNA, and histones. In both (NZB×NZW) F1 and MRL/lpr mice, estrogen treatment exacerbates the lupus disease, with augmented levels of autoantibodies against dsDNA and phospholipids as well as formation of circulating immune complexes (Grimaldi CM. 2002, Peeva E. 2000).

Murine lupus models such as NZB×NZW F1 (NZB/W F1), NZB.H-2bm12, NZB×SWR F1 (SNF1), MRL.lpr/lpr, and BXSB mice have led to a better understanding of the pathogenic mechanisms of lupus (Zhang DH. 1997, Pai SY. 2004). All of these species of mice develop immunoglobulin G (IgG) anti-dsDNA antibody, which is a characteristic of lupus, and die of uremia in early life. Among these murine lupus models, the natural course of NZB/W F1 mice is closer to human lupus than MRL.lpr/lpr and BXSB mice. The administration of the estrogen antagonist tamoxifen diminishes immune complex deposition in the kidneys and increases survival. Renal disease was evaluated by the development of albuminuria and histological changes in the kidney (Wu et al. 2000).

In NZM female mice, ERα inactivation markedly prolonged life-span, lowered proteinuria, and ameliorated glomerulonephritis but resulted in higher serum anti-dsDNA antibody levels (Svenson JL. 2008).

Empirical Evidence

Estrogen enhances anti-double-stranded DNA antibody and IgG, IgM production by PBMCs. PBMCs or B cells were cultured for 7 days with E2 (10^{-8} mol/L). Theamounts of total IgG and IgM in the supernatants were measured by ELISA. Proliferative responses PBMCs or B cells were measured by [3H]-thymidine (Kanda N. 1999).

Quantitative Understanding of the Linkage

Response-response relationship

When estrogen levels are low, T cell expansion shift toward a Th1 phenotype that produces IL-12, TNF-α, and IFN-γ. This response results in cellular immunity inducing inflammation and exacerbating cellular type autoimmune disease such as multiple sclerosis (MS) and EAE rather than SLE.

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Cunningham and Gilkeson, 2011). Treatment with low serum levels (60–100 pg/mL or 0.26–0.43 nM) of estradiol increased Th1 T-cell development in vitro by acting through an ERα mediated mechanism (Maret et al. 2003). Treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated disease, while high dose levels (>1000 pg/ml or 4.3 nM), which mimic pregnancy levels, prevented EAE onset and polarized T-cells to a Th2 phenotype in the EAE. (Bebo et al. 2001). High levels of estrogen during pregnancy have been reported to ameliorate T cell mediated diseases such as multiple sclerosis (Korn-Lubetzki et al. 1984).

Known modulating factors

The Th1/Th2 shift is one of the most important immunologic changes during the menstrual cycle and gestation. Immune activity shifts across the menstrual cycle, with higher follicular-phase Th1 cell activity and higher luteal-phase Th2 cell activity (Tierney et al. 2015). This is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses (Doria, A., et al. 2006).

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AOP ID and Title:

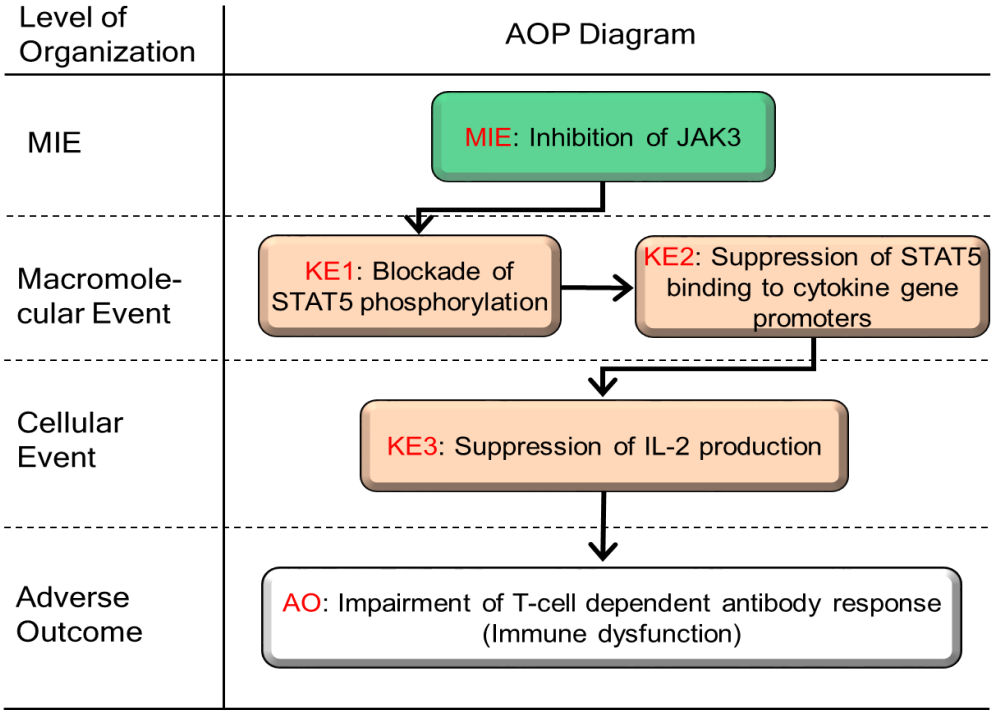
SNAPSHOT

Created at: 2020-05-18 09:23

AOP 315: Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response

Short Title: Immune dysfunction induced by JAK3 inhibition

Graphical Representation



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Status

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Abstract

Signal transduction between immune-related cells depends in many cases on cytokines and takes place via cell surface cytokine receptors as well as direct cell-to-cell interaction. Cytokines influence the movement, proliferation, differentiation, and activation of lymphocytes and other leukocytes in a variety of ways.

Some receptors for cytokines require and activation step through a Janus-kinase (JAK) Signal Transducers and Activator of Transcription (STAT) system. When cytokine binds to its specific cytokine receptors, the cytokine receptors form dimers, which more closely resemble the JAK molecules. The JAK then activates to phosphorylate adjacent cytokine receptors. STATs bind to the phosphorylated sites of the receptors and

are then phosphorylated by the activated JAK. The phosphorylated STAT is dimerized to be translocated into nucleus and bind to promoter regions of cytokine genes, which starts transcription of cytokine genes in the nucleus.

In mammals, four JAK families of enzymes (JAK1, JAK2, JAK3, TYK2) and seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6) are utilized by more than 50 cytokines and growth factors to mediate intracellular signaling. In particular, pro-inflammatory cytokines such as interferon-γ (IFN-γ), interleukin-2 (IL-2), IL-4, IL-6, IL-13, IL-21 and IL-23 have been implicated in inflammatory diseases that utilize the JAK pathway. In addition, TH2 derived cytokines, including IL-31 and thymic stromal lymphopoietin (TSLP), are ligands for murine and human sensory nerves and have a critical function that evokes itchiness. Because these cytokines also interact with JAK, several JAK-inhibitors have received a lot of attention recently as a therapeutic agent for major inflammatory diseases and pruritic diseases. However, immunotoxic events due to inhibition of the JAK pathway have yet to be examined.

This AOP focuses on the inhibition of JAK3, which is required for signal transduction by cytokines through the common gamma (γ) chain of the interleukin receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. This AOP proposes JAK3 inhibition as an MIE that leads to suppression of T cell-dependent antibody response (TDAR) as an AO. TDAR is frequently affected under immunosuppressive conditions and is a major endpoint in many preclinical immunotoxicity studies. In this proposed AOP, JAK3 selective inhibitors (e.g. PF-06651600, RB1) are stressors, blockade of STAT5 phosphorylation is KE1, suppression of STAT5 binding to the promoter regions of cytokine genes is KE2, and subsequent suppression of IL-2 production is KE3.

Background

Although there are numerous stressors that inhibit JAK3 activity, this AOP is based on immunosuppression caused by recently developed, highly selective JAK3 inhibitors PF-06651600 and RB1, about which a significant body of scientific literature has been published.

We look forward to future amendments to this AOP with up-to-date information on other stressors, which will clarify the link between inhibition of JAK activity and impairment of TDAR.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	1715	Inhibition of JAK3 (https://aopwiki.org/events/1715)	Inhibition of JAK3
	KE	1716	Blockade of STAT5 phosphorylation (https://aopwiki.org/events/1716)	STAT5 inhibition
	KE	1717	Suppression of STAT5 binding to cytokine gene promoters (https://aopwiki.org/events/1717)	Suppression of STAT5 binding
	KE	1718	Suppression of IL-2 production (https://aopwiki.org/events/1718)	Suppression of IL-2 production
	AO	1719	Impairment of T-cell dependent antibody response (https://aopwiki.org/events/1719)	Impairment, TDAR

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition of JAK3 (https://aopwiki.org/relationships/2024)	adjacent	Blockade of STAT5 phosphorylation	High	High
Blockade of STAT5 phosphorylation (https://aopwiki.org/relationships/2025)	adjacent	Suppression of STAT5 binding to cytokine gene promoters	High	High

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Suppression of STAT5 binding to cytokine gene promoters (https://aopwiki.org/relationships/2026)	adjacent	Suppression of IL-2 production	High	High
Suppression of IL-2 production (https://aopwiki.org/relationships/2027)	adjacent	Impairment of T-cell dependent antibody response	High	High

Overall Assessment of the AOP

Janus kinases (JAKs) are a family of nonreceptor tyrosine kinase and consists of four members: JAK1, JAK2, JAK3, and Tyk2 (1). All four members mediate signals initiated by cytokines through interactions with receptors for IL-2, IL-5, IL-7, IL-9, and IL-15 via the common γ chain (2). Previous studies with IL-2Rγ-null mice showed that JAK3 is related to the development of spontaneous IBD symptoms (3). Moreover, abnormal activation of JAK3 was associated with human hematological (4), indicating that a tight balance of its activity was essential for normal hematopoietic development.Janus kinases (JAKs) are a family of nonreceptor tyrosine kinase and consists of four members: JAK1, JAK2, JAK3, and Tyk2 (1). Different studies have shown that JAK3 is widely expressed in different organs (2). Previous studies with IL-2Rγ-null mice showed that JAK3 is related to the development of spontaneous IBD symptoms (3). Moreover, abnormal activation of JAK3 was associated with human hematological (4), indicating that a tight balance of its activity was essential for normal hematopoietic development.

Although JAK1, JAK2, and Tyk2 are each widely expressed, JAK3 is predominantly expressed in hematopoietic cells and is known to associate only with the common γ (γc) chain of the interleukin-2 (IL-2), IL-4, IL-7, IL-9, and IL-15 receptors (5). Homozygous mutant mice in which the JAK3 gene had been disrupted were generated by gene targeting. JAK3-deficient mice had profound reductions in thymocytes and severe B cell and T cell lymphopenia similar to severe combined immunodeficiency disease (SCID), and the residual T cells and B cells were functionally deficient. Thus, JAK3 plays a critical role in γc signaling and lymphoid development.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Sex Applicability

Sex	Evidence
Mixed	High

This proposed AOP involves inhibition of JAK activity leading to suppression of TDAR and is not dependent on life stage, sex, or age. Since JAK3 inhibitors (PF-06651600, RB1) are currently under a phase 2 clinical evaluation to treat rheumatoid arthritis, the AOP appears to be applicable to all life stages. Since JAK3 inhibitor-induced outcomes in humans are mimicked by similar responses in a variety of animal models, including non-human primates and rodents, immunosuppression induced by inhibition of JAK3 activity is considered to occur across a variety of mammalian species.

Essentiality of the Key Events

MIE and later events: JAK3-knockout (KO) mice

JAK3 was initially identified (1,2) in studies to identify the JAK family member that was involved in the signaling of a group of cytokines that shared in common the utilization of the γc chain first identified in the interleukin 2 (IL-2) receptor complex. It was subsequently demonstrated that JAK3 physically associates with the γc chain and is activated in a receptor complex that also contains JAK1, which associates with the ligand specific alpha or beta chain of the receptors (3). JAK3 is somewhat unique within the JAK family in that it is predominantly expressed in

hematopoietic cells and is only activated in the responses to cytokines that use the γc chain (4). The phenotype of the JAK3 deletion mice was quite striking and consisted of a range of deficiencies which collectively constituted SCID (5,6). At the same time, two groups identified individuals that lacked JAK3 and exhibited somatically acquired SCID (7,8). One of the most striking components of the phenotype is the dramatic reduction seen in both the T and B cell lineages. Comparable reductions are seen in mice that lack IL-7 (9), the IL-7 receptor alpha chain (10), or the γc chain. In spite of the reduced numbers, the cells that do develop are phenotypically normal. These results are consistent with the hypothesis that activation of JAK3 give it a critical role in the expansion but not the differentiation of early lymphoid lineage-committed cells. In addition to the reduced numbers, the differentiated lymphoid cells that are generated fail to respond to the spectrum of cytokines that utilize the γc chain and activate JAK3 normally.

Stressor: B6.Cg-Nr1d1tm1Ven/LazJ mouse

The B6.Cg-Nr1d1tm1Ven/LazJ mouse line harbors a spontaneous mutation in JAK3, which generates an SCID phenotype with an inability to generate antigen-independent professional cytokine-producing innate lymphoid cells (ILCs). Mechanistically, JAK3 deficiency blocks ILC differentiation in the bone marrow at the ILC progenitor (ILCP) and the pre-NK cell progenitor (pre-NKP). Similar phenomenon was further demonstrated by the pan-JAK inhibitor tofacitinib and specific JAK3 inhibitor PF-06651600. Both JAK-inhibitors impair the ability of human intraepithelial ILC1 (iILC1) to produce IFN-γ, without affecting ILC3 production of IL-22. Both inhibitors impaired the proliferation of iILC1 and ILC3 and differentiation of human ILC in vitro. These findings indicate that JAK3 deficiency blocks innate lymphoid cell development (11).

KE1 and later events: STAT5-KO mice

STAT5 plays a major role in regulating vital cellular functions such as proliferation, differentiation, and apoptosis of hematopoietic and immune cells (12,13). STAT5 is activated by phosphorylation of a single tyrosine residue (Y694 in STAT5) and negatively regulated by dephosphorylation. A wide variety of growth factors and cytokines can activate STAT5 through the JAK-STAT pathway. The activation of STAT5 is transient and tightly regulated in normal cells (14).

The following phenotypes are observed in STAT5-KO mice:

The transcription factor STAT5 is expressed in all lymphocytes and plays a key role in multiple aspects of lymphocyte development and function (15). STAT5 was initially identified as a transcription factor activated by prolactin in mammary gland epithelial cells (16,17). Subsequent studies identified STAT5 binding activity in T cells (18), and it was later established that STAT5 was expressed in multiple cell types and activated by a number of cytokines, including the common gamma chain (γc)-dependent cytokines interleukin 2 (IL2), IL4, IL7, IL13, and IL15 (19).

STAT5 in T-cell development

The observation that STAT5 is activated by multiple cytokines in T cells suggested that it might play a critical role in the development or function (or both) of these cells. Disruption of Stat5a or Stat5b genes alone resulted in relatively modest phenotypes; for example, Stat5a-/- mice had defects in mammary gland development and lactation while Stat5b-/- mice had defects in response to growth hormone in male mice and natural killer cell proliferation (20,21). To determine whether combined deletion of Stat5a and Stat5b might result in more profound immunodeficiencies, subsequent studies deleted the first coding exons of both Stat5a and Stat5b. This intervention resulted in the production of truncated forms of STAT5a and STAT5b that acted as functional hypomorphs. These mice too had surprisingly mild defects in lymphocyte development, although T cells were grossly dysfunctional, as they could no longer proliferate in response to IL2 (22,23). Finally, complete deletion of Stat5a and Stat5b using Cre-LoxP approaches demonstrated that STAT5a and STAT5b are absolutely required for lymphocyte development, as Stat5a/b-/- mice had profound blocks in lymphocyte development, which mimicked that observed in Il7r-/- mice (24,25). These studies definitively demonstrated that the STAT5 hypomorph mice retained significant STAT5 function.

Weight of Evidence Summary

Biological Plausibility

T-cell development is mainly regulated by JAK-STAT system, and JAK3 deficiency in T cells is known to induce multiple types of immunosuppression, including T cell-dependent antibody response (TDAR).

JAK3-deficient mice had profound reductions in thymocytes and severe B cell and T cell lymphopenia similar to SCID disease, and the residual T cells and B cells were functionally deficient (10).

Mice lacking JAK3 also showed a severe block in B cell development at the pre-B stage in the bone marrow. In contrast, although the thymuses of these mice were small, T cell maturation progressed relatively normally. In response to mitogenic signals, peripheral T cells in JAK3-deficient mice did not proliferate and secreted small amounts of IL-2. These data demonstrate that JAK3 is critical for the progression of B cell development in the bone marrow and for the functional competence of mature T cells (5).

Furthermore, the abnormal architecture of lymphoid organs suggested the involvement of JAK3 in the function of epithelial cells. T cells developed in the mutant mice did not respond to either IL-2, IL-4, or IL-7 (26).

Specific JAK3 inhibitor PF-06651600 or RB1, which selectively inhibited JAK3 with an over 100-fold preference over JAK2, JAK1, and TYK2 in the kinase assay, displayed reduced inflammation and associated pathology in collagen-induced-arthritis mice. Importantly, with PF-06651600 or RB1 administration, pro-inflammatory cytokines and JAK3 and STATs phosphorylation decreased in mice, suggesting that the inhibition of JAK3/STAT signaling was closely correlated with induction of multiple types of immunosuppression, including TDAR .

Quantitative Consideration

KER1 (MIE=>KE 1)

Interleukin 2 (IL-2) activated STAT5 via distinct pathways (30).

IL-2 have been demonstrated to stimulate STAT5 and induce tyrosine phosphorylation of STAT5. Treatment of highly selective JAK3 inhibitors

(PF-06651600 or RB1) treatment clearly suppresses the complex formation of STAT5 in the nucleus.

Highly-selective JAK3 inhibitor RB1 inhibited the phosphorylation of STAT5 elicited by IL-2 at IC50 value of 31 nM in the raw peripheral blood mononuclear cells (PBMCs) of humans. PBMCs were isolated from the buffy coats of healthy volunteers using density gradient centrifugation on Lymphoprep. Cells were cultured in complete RPMI 1640 medium (containing 10% foetal bovine serum, 100 mg/ml streptomycin and 100 U/ml penicillin) plus 10 µg/ml lectin phytohemagglutinin (PHA) for 3 days and then treated with either recombinant human IL-6 (400 ng/ml), recombinant human IL-2 (100 ng/ml), or recombinant human GM-CSF (50 ng/ml) at 37 °C for 20 min. To terminate the stimulation, cells were fixed with Lyse/Fix Buffer and then incubated with 100% methanol for 30 minutes; cells were incubated with anti-pSTAT3 and anti-CD4 Abs, or anti-pSTAT5 and anti-CD4 Abs at 4 °C overnight, washed twice with PBS, and analysed with an flow cytometer (31).

Fluorescence intensity for phospho-STAT5 in CD3-positive lymphocytes increased upon incubation of peripheral blood with IL-2. Peficitinib inhibited STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 124 nM (101 and 147 nM for two rats). Additionally, the effect of peficitinib on IL-2 stimulated STAT5 phosphorylation in human peripheral T-cells was evaluated. Paralleling results in rats, the fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes increased in human peripheral blood after adding IL-2, but peficitinib inhibited STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 127 nM in human lymphocytes (26).

KER2 (KE1 =>KE 2)

IL-2 activated STAT5 (30).

IL-2 have been demonstrated to stimulate STAT5 and induce tyrosine phosphorylation of STAT5. These IL-2-induced STATs have an identical DNA binding specificity and immunoreactivity.

KER3 (KE2 =>KE 3)

IL-2 activated STAT5 (30)

IL-2 have been demonstrated to stimulate STAT5 and induce tyrosine phosphorylation of STAT5. These IL-2-induced STATs have an identical DNA binding specificity and immunoreactivity.

Gel mobility shift assay showed that IL-2 activation induced STAT5 dimerization and DNA binding to gamma interferon-activated site (GAS) motif in IL-2 promoter region (32).

KER4 (KE3 =>AO)

As for IL-2 and antibody production, in vitro T-cell-induced polyclonal B cell activation to produce antibody was inhibited with anti-IL-2 and anti-IL-2R antibodies (33). In addition, cynomolgus monkeys treated with CsA showed suppression of IL-2 and TDAR using sheep red blood cells with a dose dependent manner (34).

In the human T-B cell co-culture stimulated with anti-CD3 monoclonal antibody, CNIs of FK506 and CsA lowered the levels of T-cell cytokines including IL-2 and IL-4 and inhibited IgM and IgG productions with a dose-dependent manner (35).

These results show the quantitative relationships between the inhibition of IL-2 by specific antibodies or CNI and suppression of antibody production.

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Appendix 1

List of MIEs in this AOP

Event: 1715: Inhibition of JAK3 (https://aopwiki.org/events/1715)

Short Name: Inhibition of JAK3

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response (https://aopwiki.org/aops/315)	MolecularInitiatingEvent

Biological Context

Level of Biological Organization
Molecular

List of Key Events in the AOP

Event: 1716: Blockade of STAT5 phosphorylation (https://aopwiki.org/events/1716)

Short Name: STAT5 inhibition

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response (https://aopwiki.org/aops/315)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Event: 1717: Suppression of STAT5 binding to cytokine gene promoters (https://aopwiki.org/events/1717)

Short Name: Suppression of STAT5 binding

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response (https://aopwiki.org/aops/315)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Event: 1718: Suppression of IL-2 production (https://aopwiki.org/events/1718)

Short Name: Suppression of IL-2 production

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response (https://aopwiki.org/aops/315)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

List of Adverse Outcomes in this AOP

Event: 1719: Impairment of T-cell dependent antibody response (https://aopwiki.org/events/1719)

Short Name: Impairment, TDAR

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response (https://aopwiki.org/aops/315)	AdverseOutcome

Biological Context

Level of Biological Organization
Individual

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2024: Inhibition of JAK3 leads to STAT5 inhibition (https://aopwiki.org/relationships/2024)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response (https://aopwiki.org/aops/315)	**adjacent**	**High**	**High**

Relationship: 2025: STAT5 inhibition leads to Suppression of STAT5 binding (https://aopwiki.org/relationships/2025)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response (https://aopwiki.org/aops/315)	**adjacent**	**High**	**High**

Relationship: 2026: Suppression of STAT5 binding leads to Suppression of IL-2 production

(https://aopwiki.org/relationships/2026)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response (https://aopwiki.org/aops/315)	**adjacent**	**High**	**High**

Relationship: 2027: Suppression of IL-2 production leads to Impairment, TDAR (https://aopwiki.org/relationships/2027)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response (https://aopwiki.org/aops/315)	**adjacent**	**High**	**High**

厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）

OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

令和元年度 分担研究報告書

遺伝毒性の AOP 開発

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研究要旨

有害性発現経路（AOP: Adverse Outcome Pathway）開発にあたり、同 AOP への組み込みを想定したエピジェネティック毒性試験法「FLO assay」の妥当性を検証する研究を実施している。本年度は国際がん研究機関でグループ 2B（Possibly carcinogenic to humans）と分類されるかび毒オクラトキシン A の類縁化合物であり発がん性の懸念があるシトリニンを被検物質として FLO assay を行なった。その結果、シトリニンはオクラトキシン A と同様のエピジェネティック作用を有する可能性が明らかとなった。本結果は、発がん性予測の精緻化に FLO assay が利用できる可能性を示唆している。

A. 研究目的

現在、経済協力開発機構（OECD）において、毒性評価に有害性発現経路（AOP）の活用の検討が進められている。発がんに係わるエピジェネティック制御の攪乱を同AOPに組み込む有用性は高いと考えられる。

本研究では、研究分担者が開発したエピジェネティック変異原検出系「FLO assay」を用いて、国際がん研究機関（IARC）でグループ2B（Possibly carcinogenic to humans）と分類されるかび毒オクラトキシンAの類縁化合物と考えられ発がん性が危惧されるかび毒シトリニンのエピジェネティック作用の評価を行い、発がん性AOPへのFLO assay活用の妥当性を検証した（Fig. 1）。

B. 研究方法

B.1. 酵母株

出芽酵母 *Saccharomyces cerevisiae* YPH250株は、University of California at Berkeley, CA, USAより入手した。使用した株および関連情報はTable 1および2に示す。

B.2. 使用した化学物質

シトリニンは和光純薬工業（株）より購入した。シトリニンは現在IARCでグループ3（Not classifiable as to its carcinogenicity to humans）と分類されている。

B.3. 培地

Synthetic Dextrose (SD) -Trp/-Uraもしくは-Trp/-Ura/-His最少液体培地は以下の通りに調製した。MilliQ 水に -Trp/-Ura DO Supplement (Clontech, USA) 0.072%、もしくは -Trp/-Ura/-His DO Supplement (Clontech, USA) 0.07%、Yeast Nitrogen Base w/o Amino Acids (Becton and Dickinson, USA) 0.67%を加えオートクレーブ (121℃ 20 min) 後、20% グルコース (Wako, Osaka, Japan)を終濃度が2.0%になるよう加えて4℃で保存した。

B.4. 凝集試験

SD -Trp/-Ura液体培地において、各被検物質存在下もしくは非存在下にて30度で対数増殖期中期から定常期初期まで振盪培養を行い、凝集レベルを相対的凝集活性として測定した。相対的凝集活性（Relative flocculation activity）は、培養液中の透明領域の高さ（T）と培養液全体の高さ（C）を測定し、次式を用いて算出した。

Relative flocculation activity = 100 × (T/C)

B.5. *FLO1*レポーターアッセイ

使用した株およびレポータープラスミドはTable 1および2のとおりである。SD -Trp/-Ura/-His液体培地において対数増殖期後期まで培養した酵母細胞を回収後洗浄し蛍光（Excitation, 485 nm; Emission, 535 nm）を測定した。測定にはTriStar² LB 942 (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany)を使用した。なお、蛍光強度は濁度（OD600）で補正した。

B.6. In vitroメチレーションアッセイ

基質にプラスミドpUC19を用いてM.SssIメチラーゼ（New England Biolabs、Ipswich、MA）に対するシトリニンの作用を検討した。メチル化反応は37℃で２時間行い、その後メチル化感受性制限酵素HpaII（New England Biolabs）で反応物を消化し、2%Tris-borate EDTAアガロースゲルで分析した。

B.7. 統計処理

一元配置分散分析を行った後、Dunnett's *post hoc* testを用いて有意差検定を行った。測定値は標準誤差で表示した。

（倫理面への配慮）

本研究は微生物を用いた研究であり、当該項目は非該当に相当する。

C. 研究結果

C.1. シトリニンが *FLO1* レポーター活性に及ぼす影響

酵母の凝集遺伝子 *FLO1* の発現はエピジェネテッィク制御を受ける。そこで FLO assay としてまずシトリニン（0.5-4.0 μM）が *FLO1* 発現におよぼす影響を、*FLO1* プロモーター誘導性レポーター活性を指標に検討した。その結果、シトリニンは Empty-vector control strain（コントロール株）と *DNMT*yeast（DNA メチル化酵素（*DNMT*）遺伝子形質転換酵母）の両株において *FLO1* レポーター活性を濃度依存的に抑制した。この抑制効果は、レポータープラスミド pFIGSTpA（DNA メチル化低感受性となる CpG-reduced *FLO1*プロモーターを有する）を有する株では減弱した（Fig. 2）。

C.2. シトリニンが凝集に及ぼす影響

*DNMT*yeast は *FLO1* 発現が亢進し誘導型凝集性を示す。FLO assay として次にシトリニンが凝集性に及ぼす影響を *DNMT* yeast で検討した。その結果、シトリニン（0.5-2.0 μM）の濃度依存的に凝集性が抑制されることが明らかとなった（Fig. 3）。

C.3. DNA メチル化酵素に及ぼすシトリニンの影響

微生物由来の DNA メチル化酵素 *M*.SssI は、ヒト DNMT のアナログである。シトリニン（4, 40 μM）存在下における *M*.SssI の酵素活性を *in vitro* で検討した結果、シトリニンの濃度依存的に同 DNA メチル化活性が抑制される可能性を認めた（Fig. 4）。

D. 考察

本研究では、IARCでグループ2B（Possibly carcinogenic to humans）と分類されるオクラトキシンAの類縁化合物かび毒シトリニンを被検物質として、エピジェネティック変異原性をFLO assay（*FLO1*レポーターアッセイと凝集試験）を主要な指標として評価した。その結果、シトリニンは*FLO1*レポーター活性と凝集性を濃度依存的に抑制した。本結果は、DNAメチル化阻害作用を有すると考えられるオクラトキシンAによる両抑制作用と同様の作用がシトリニンにも認められたことを意味する。さらに本研究から、DNAメチル化低感受性*FLO1*プロモーター活性に対してはシトリニンによる顕著な抑制作用は確認できず、*in vitro*のDNAメチルレーションにおいては同かび毒存在下においてDNAメチル化酵素活性が阻害される可能性も明らかとなった。以上の結果は、シトリニンがオクラトキシンAと同様にDNAメチル化阻害を作用機序（Mode of Action: MoA）とするエピジェネティック変異原であることを示唆しており、げっ歯類に対する発がん作用に同作用が関与している可能性を示唆している。両かび毒がげっ歯類において共に発がん作用を有している事実とも矛盾はない。

E. 結論

本研究において、発がん性が危惧されるかび毒シトリニンは可塑的にエピジェネティック制御を受ける酵母凝集遺伝子*FLO1*の転写レベルを抑制することが明らかとなった。また、同抑制メカニズムがDNAメチル化阻害に起因する可能性も見出した。これら一連の結果は、シトリニンがDNAメチル化阻害を機序とするエピジェネティック変異原である可能性を示唆するものであり、FLO assayが化学発がんの予測精緻化に活用できる可能性を示している。

F. 研究発表

F.1. 論文発表

1. Sugiyama, K., Furusawa, H. and Honma, M: Detection of epigenetic effects of citrinin using a yeast-based bioassay, *Mycotoxin Res.* 2019;**35,** 363-36. doi: 10.1007/s12550-019-00361-z.

F.2. 学会発表

1. Sugiyama, K., Furusawa, H., Kinoshita, M., Takino, M. and Honma, M: Detection of epigenotoxicity of mycotoxin ochratoxin A with *DNMT* yeast, 47th Meeting of the European Environmental Mutagenesis and Genomics Society (2019). P. 76, Poster presentation, 5-21-2019, Rennes, France.

G．知的財産権の出願・登録状況

G.1. 特許取得

なし

G.2. 実用新案登録

なし

G.3.その他

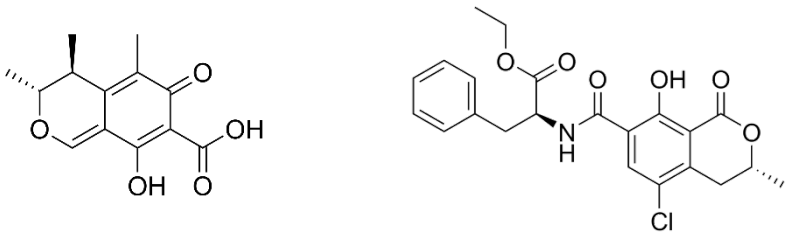
なし

Table 1 使用したプラスミド

Plasmid	Description
pY2CThD1	pYES2/CT harboling human *DNMT1* cDNA
pY3CThD3B	pYES3/CT harboling human *DNMT3B* cDNA
pYES2/CT	*E.coli- Saccharomyces* shuttle plasmid, carrying *GAL1* promoter, 2μ-type, *URA3* marker
pYES3/CT	*E.coli- Saccharomyces* shuttle plasmid, carrying *GAL1* promoter, 2μ-type, *TRP1* marker
pF1GS	*FLO1-GFP* . Parent: p313eGFP
pF1GSTpA	CpG reduced *FLO1-GFP* . Parent: p313eGFP
p313eGFP	pRS313 (*CEN*-type, *HIS3* marker) harboling a GFP variant

Table 2 使用した酵母株

Strain	Genotype	Plasmid	Name
YPH250	*MATa trp1- Δ1 his3- Δ200 leu2- Δ1 lys2-801 ade2-101 ura3-52*	pYES2/CT, pYES3/CT	empty-vector control strain
YPH250	*MATa trp1- Δ1 his3- Δ200 leu2- Δ1 lys2-801 ade2-101 ura3-52*	pY2CThD1, pY3CThD3B	*DNMT* yeast
YPH250	empty-vector control strain	pF1GS	
YPH250	empty-vector control strain	pF1GSTpA	
YPH250	empty-vector control strain	p313eGFP	
YPH250	*DNMT* yeast	pF1GS	
YPH250	*DNMT* yeast	pF1GSTpA	
YPH250	*DNMT* yeast	p313eGFP	



シトリニン　　オクラトキシン A

Fig. 1
シトリニンとオクラトキシン A の構造

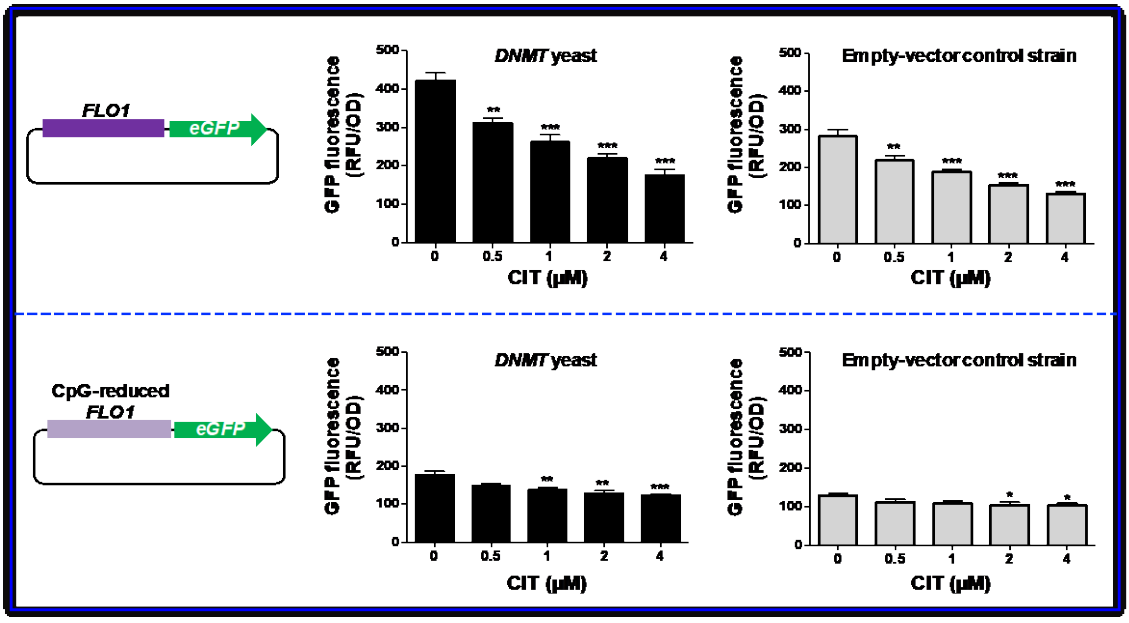


Fig. 2

FLO1 レポーター活性に対するシトリニン（CIT）の効果　左記のプラスミドマップは使用したプラスミドを示す

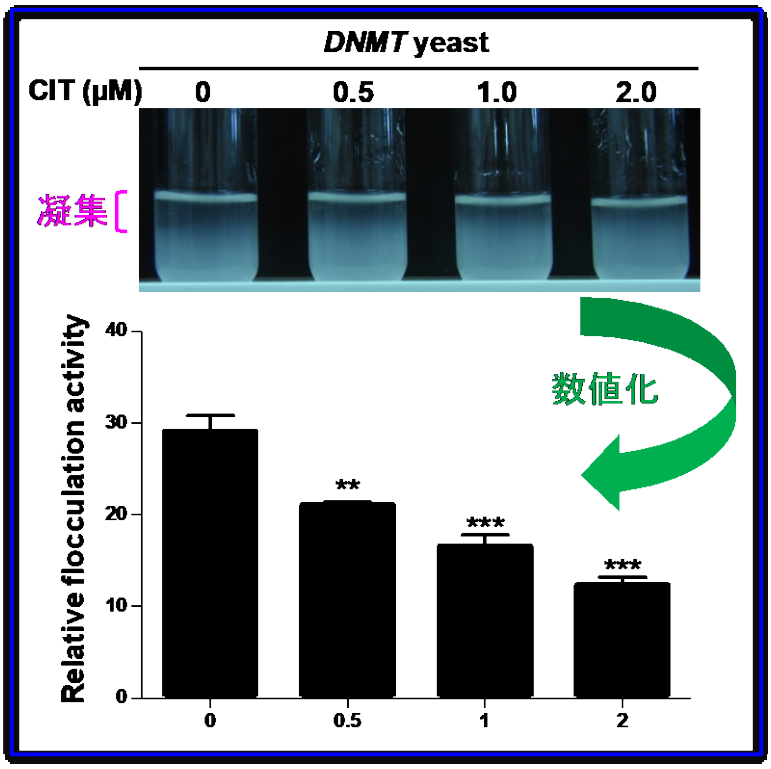


Fig. 3

凝集性に及ぼすシトリニン（CIT）の作用

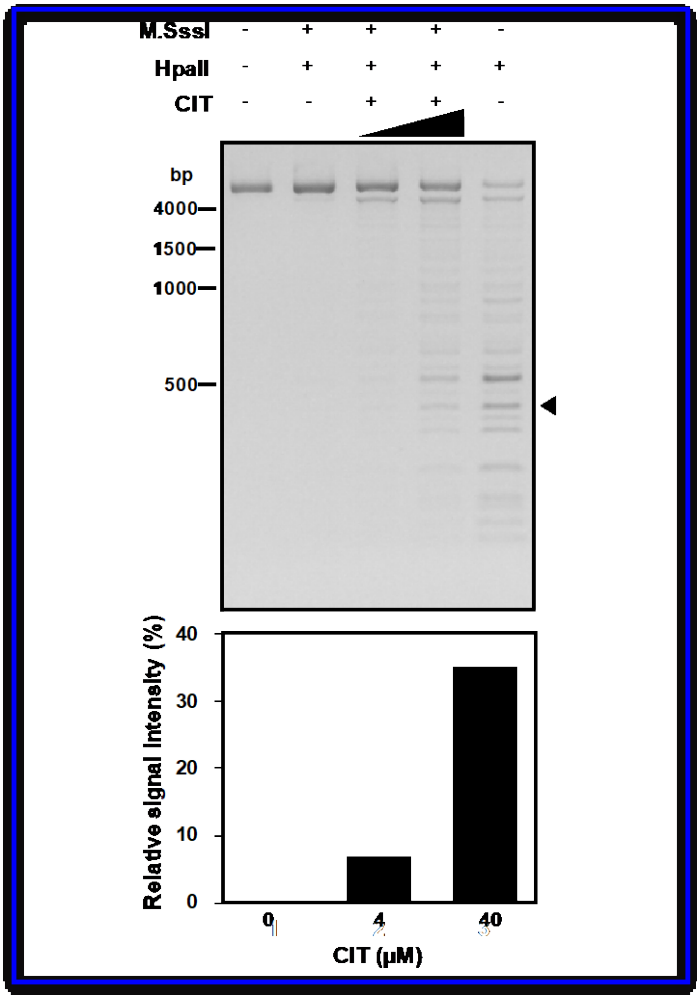


Fig. 4

シトリニン（CIT）が *M*.SssI 活性に及ぼす影響　下部のグラフは、ゲル写真における黒三角の 404bp のバンド強度を数値化し CIT による *M*.SssI への影響を数値化した結果

厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）
OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

令和元年度 分担研究報告書

腎障害の分子メカニズムに関する研究

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研究要旨

代償性反応は外来物質の暴露に対して種々の臓器に生じる生理現象である。AOP 開発プログラムにおいて、代償性反応に関する情報は各 Key Event の関連性を理解するために有用であるとされており、安全性研究者による代償性機構の解明研究は今後益々重要になるものと考えられる。腎臓は化学物質による毒性の主要な標的臓器である。腎臓の代償性反応には 2 つの機構が存在し、障害部位で生じる尿細管再生および非障害部位で生じる代償性肥大が知られる。本研究では腎臓に内在する 2 つの代償性機構を解明することを目的とした。前年度から本年度にかけては、非障害部位における代償性肥大の分子機構を解明するため、10 週齢の雌雄 F344 ラットに片側腎摘出を施し、残存腎において mRNA および microRNA（miRNA）マイクロアレイならびに mRNA-miRNA 統合解析を実施した。その結果、雌雄ともに代償性肥大には転写因子である Forkhead box M1 を介した細胞増殖が寄与しており、その下流因子の発現調節には数種類の miRNA が関与していることが明らかとなった。引き続き、障害部位における尿細管の再生メカニズムを明らかにするため、急性腎障害モデルラットを用いた先行研究の再生尿細管における mRNA マイクロアレイのデータを再解析し、再生機構に関わると考えられる候補因子を抽出した。今後はこれらの因子の再生尿細管における発現動態を免疫組織学的に解析するとともに、腎線維化モデルラットを作製して線維化病変内の尿細管における発現も併せて解析し、尿細管の再生機構およびその破綻に関連する因子を探索する予定である。

A. 研究目的

代償性反応は外来物質の暴露に対して種々の臓器に生じる生理現象である。化学物質の安全性評価において、化学物質暴露により生体あるいは細胞に生じた変化が代償性反応であった場合は有害事象とは判断されないため、代償性機構に対する理解は毒性発現機序と同様に極めて重要である。Organisation for Economic Co-operation and Development（OECD）によるAdverse Outcome Pathway（AOP）開発プログラムにおいても、代償性反応に関する情報は各Key Eventの関連性を理解するために有用であり、Key Event Relationshipの項目に記載することが推奨されている。よって、安全性研究者による代償性機構の機序解明研究の重要性は今後益々高まるものと考えられる。

腎臓は化学物質による毒性の主要な標的

臓器であり、その代償性反応として大きく2つの機構が知られている。腎毒性物質による障害はネフロン単位で生じることから、障害を受けたネフロンでは代償性反応として尿細管再生が生じ、非障害ネフロンではいわゆる代償性肥大が生じる。本研究ではこれらの2つの代償性反応の分子機構を解明することを目的とした。腎毒性発現機序には種々の様式があることに対し、代償性反応は毒性機序に関わらず共通して生じる現象であることから、本研究により腎毒性機序に依存しない新たな腎毒性評価分子を抽出することも期待できる。

本年度では前年度に引き続き、非障害部位における代償性肥大の分子機構を解明するため、片側腎摘出モデルラットを用いてmRNAおよびmicroRNA（miRNA）マイクロアレイならびにmRNA-miRNA統合解析を実施した。さらに障害部位における尿細管の再生メカニズムに関与する因子を探索するため、急性腎障害モデルラットを用いた先行研究の再生尿細管のmRNAマイクロアレイのデータを再解析し、候補因子を抽出した。

B. 研究方法

腎障害時に非障害領域で生じる代償性肥大の分子機構を解明するため、10週齢の雌雄F344ラットをそれぞれ6群（n=5）に配した後、イソフルラン深麻酔下にて片側腎（左腎臓）摘出術を施し、処置後1, 2, 3および7日に安楽殺した。対照群にはSham処置として開腹術のみ実施し、処置後3および7日に同様に安楽殺した。細胞増殖活性の評価を実施するため、全ての動物について安楽殺の2時間前にbromodeoxyuridin（BrdU）を100 mg/kg体重の用量で単回腹腔内投与した。安楽殺時に右腎臓を採材して重量を測定した後、一部を10%中性緩衝ホルマリン液にて固定し、残りの組織を液体窒素で瞬間凍結させ、-80℃にて保存した。ホルマリン固定サンプルを用いて定法に従いパラフィン包埋および薄切を行い、BrdU免疫染色およびPeriodic acid-Schiff 染色（PAS）の二重染色を施して、近位曲尿細管、近位直尿細管および遠位尿細管におけるBrdU陽性細胞率を算出した。瞬間凍結サンプルからRNeasy Mini Kitにより total RNA を抽出し、RT-qPCR およびmRNAマイクロアレイに供した。また雄については凍結サンプルよりmiRNeasy Mini Kitを用いてtotal RNAを抽出し、miRNAマイクロアレイに供した。mRNAおよびmiRNAのマイクロアレイ解析は、BrdU陽性細胞率ならびにcyclin E1およびcyclin B1のqPCR解析の結果に基づき、処置後2日および対照群のサンプルを用いて実施した。マイクロアレイにおいて変動のあったmRNAについては、Ingenuity Pathways Analysis（IPA）を用いてパスウェイ解析を実施した。

また、化学物質誘発急性腎障害モデルラットにおける代償性肥大の分子機構を探索する目的で、6週齢の雄性SDラットに125 mg/kgのFolic acidあるいは媒体である生理食塩水を単回腹腔内投与した。投与後1日に腎臓を採材し、左腎臓の皮質を肉眼的に分離して液体窒素で瞬間凍結させ、-80℃にて保存してqPCR解析に供した。右腎臓の組織を10%中性緩衝ホルマリン液にて固定し、上記と同様に組織標本を作製してHE染色およびKi67免疫染色を行った。

尿細管の再生メカニズムに寄与する因子を探索するため、先行研究（Matsushita et al., Toxicol Sci. 2018;165(2):420-430）における再生尿細管のmRNAマイクロアレイデータを再解析した。先行研究の研究方法を以下に簡潔に示す。10週齢の雌性F344ラットの左

腎臓に120分あるいは90分の虚血処置を施し、再灌流後それぞれ3および7日に安楽殺した。病理組織学的解析において、処置後3日には再生初期、7日には再生後期の再生尿細管がみられたため、凍結標本からレーザーマイクロダイセクションによりそれぞれの尿細管を採取し、mRNAマイクロアレイを実施して遺伝子発現プロファイルを対照群（Sham処置群）より採取した正常尿細管と比較した。

（倫理面への配慮）

動物の数は最小限にとどめ、実験は国立医薬品食品衛生研究所の実験動物取扱い規定に基づき、動物の苦痛を最小限とするよう配慮して行った。

C. 研究結果

片側腎摘出ラットにおける残存腎のBrdU免疫染色では、処置後2日以降において対照群と比して近位尿細管曲部、近位尿細管直部および遠位尿細管におけるBrdU陽性細胞率の有意な増加あるいは増加傾向がみられた（Figure 1）。mRNAのqPCR解析においては、雌雄ともに細胞増殖に関わるcyclin E1およびcyclin B1の発現が処置後2日以降に対照群と比して有意な増加あるいは増加傾向を示した（Figure 2）。一方、雌雄ともに細胞周期停止および細胞肥大に関与するtransforming growth factor (TGF)-β1の発現に変動はみられなかった（Figure 2）。

mRNAマイクロアレイ解析では、対照群に比して雄では320遺伝子、雌では233遺伝子の発現が変動していた。IPAを用いたNew Comparison Analysisの結果、雌雄ともに細胞増殖に関わるCanonical Pathwayの活性化が認められた（Figure 3）。細胞増殖を制御する上流因子を検索するため、Upstream Regulator Analysisを実施した結果、転写因子であるForkhead box M1 (FOXM1)が細胞周期関連遺伝子の上流因子として抽出された（Table 1）。qPCRにてFOXM1の発現動態を確認した結果、雌雄ともに処置後2日以降に対照群と比して有意な発現増加が認められた（Figure 4）。

雄ラットにおけるmiRNAマイクロアレイ解析では、対照群と比して処置群において9個のmiRNA発現が低下していた（Table 2）。mRNA-miRNA統合解析では、miR-653-5p, miR-31-5pおよびmiR-9-3pはFOXM1の下流因子の制御に直接的に関与していること、さらにmiR-1843a-5p, miR-194-3pおよびmiR-31-5pはトランスフェリン受容体1を介して間接的にFOXM1の下流因子の制御に寄与していることが示唆された（Figure 5）。

腎障害物質であるFolic acidを投与したラット腎臓では、病理組織学的に髄質外層内帯の尿細管に壊死が認められ、非障害部位である皮質においては、Ki67に陽性を示す尿細管が増加した（Figure 6A）。非障害部位の皮質組織を用いたqPCR解析では、Folic acid投与群において対照群と比してcyclin E1およびFOXM1の発現が有意に増加していた一方、TGF-β1の発現に差は認められなかった（Figure 6B）。

尿細管の再生メカニズム研究では、腎障害モデルラットを用いた先行研究における再生尿細管のmRNAマイクロアレイのデータを再解析し、文献調査の結果に基づいて尿細管の再生機構に関与する候補因子として10個の因子（Survivin, RUNX1, CD44, Osteoactivin, Adrenoceptor alpha 1D, Kim1, SOX9, Osteopontin, CD133およびPAX2）を抽出した。

D. 考察

腎臓の代償性肥大には細胞の増数（過形成）および細胞の大きさの増大（肥大）が関与するとされている。これまでの報告により、代償性肥大の作用様式には種差、系統差および性差があることが示されているため、本研究では毒性試験で汎用される性成熟した雌雄のラットを用いて実験を行った。細胞周期のG1/S期あるいはG2/M期移行が生じた際には細胞が分裂して過形成が生じ、反対にG1/S期あるいはG2/M期でarrestが生じた場合には細胞周期が停止して肥大が生じることが知られている。TGF-β1はG1/S arrestを誘導して肥大に寄与することが知られているが、本実験結果では雌雄ともにTGF-β1の発現変動に片側腎摘出の影響はみられなかった。一方、片側腎摘出により雌雄ともに残存腎のBrdU陽性細胞率が上昇し、それぞれG1/S期およびG2/S期移行に関与するcyclin E1およびcyclin B1のmRNA発現上昇がみられた。また、mRNAマイクロアレイのデータを用いたパスウェイ解析により、雌雄ともに細胞増殖に関与する経路が活性化していた。以上の結果より、雌雄ともに腎代償性機構には細胞肥大ではなく過形成が寄与していることが示唆された。

Upstream Regulator Analysisの結果、FOXM1が細胞増殖の上流因子であることが示唆された。FOXM1は腸管等の細胞分裂の活発な正常組織や種々の腫瘍組織で高発現することが知られている。FOXM1はG1/S期およびG2/M期移行を促進することで細胞分裂を引き起こすため、FOXM1は腎臓における代償性肥大の作用様式を決定づける因子である可能性が考えられた。

miRNAマイクロアレイの結果、9種類のmiRNAの発現低下が認められた。mRNA-miRNA統合解析では、miRNAがFOXM1の下流因子の制御に直接的あるいはトランスフェリン受容体1を介して間接的に関与していることが示唆された。腎腫瘍を含む種々の腫瘍組織において、細胞増殖活性の増加に伴って鉄の要求量が上昇し、トランスフェリン受容体1依存性の鉄の取込みが亢進することが報告されている。本研究結果より、腎臓の代償性肥大においても、トランスフェリン受容体1を介した鉄の取込みの亢進が生じていることが示唆された。

Folic acidを用いた急性腎障害モデルラットの腎臓において、非障害領域である皮質にKi67陽性を示す尿細管が増加し、FOXM1およびcyclin E1のmRNA発現が亢進した。以上の結果より、化学物質誘発腎障害における代償性機構においても、FOXM1を介した細胞増殖の亢進が寄与していることが考えられた。

腎臓の代償性反応として、障害部位では尿細管再生が生じる。腎障害が生じた際、障害を受けたネフロンでは壊死した尿細管に隣接する尿細管が脱分化して再生尿細管に形質転換し、活発に増殖した後に再分化して組織が修復され、腎機能は回復する。一方、この尿細管の再生機構が何らかの原因で破綻した場合は、不可逆的な線維化が生じ慢性病変へと進展する。よって、尿細管の再生機構およびその破綻に寄与する因子を同定することは、尿細管再生機構の破綻をKey Eventとした腎線維化のAOP開発の支援に繋がることが期待される。本年度は先行研究のmRNAマイクロアレイデータを再解析し、再生尿細管に発現する10種類の因子を尿細管再生に寄与する候補因子として抽出した。来年度にはこれらの因子の再生尿細管における発現動態を免疫組織化学的に解析するとともに、腎線維化モデルラットを作製し、線維化病変内の再生機構の破綻した尿細管

におけるこれらの因子の発現も併せて解析する予定である。

E. 結論

腎臓の非障害部位における代償性肥大にはFOXM1を介した細胞増殖が寄与しており、その下流因子の発現調節にはmiRNAが寄与していることが明らかとなった。今後は尿細管の再生メカニズムを明らかにし、腎障害あるいは腎線維化のAOP開発に資する知見を提供したいと考える。

F. 研究発表

F.1. 論文発表

1) Matsushita K, Toyoda T, Yamada T, Morikawa T, Ogawa K. Comprehensive expression analysis of mRNA and microRNA for investigation of compensatory mechanisms in the rat kidney after unilateral nephrectomy. Journal of Applied Toxicology. in press.

F.2. 学会発表

該当なし

G. 知的財産権の出願・登録状況

G.1. 特許取得

該当なし

G.2. 実用新案登録

該当なし

G.3.その他

該当なし

Table 1. 片側腎摘出後の残存腎における Upstream Regulator Analysis

Upstream Regulator	Fold Change	Activation z-score	p-value of overlap
FOXM1	2.177	4.699	2.75E-25
E2F1	4.039	3.785	3.46E-21
MYBL2	2.887	3.107	9.04E-13

Table 2. 片側腎摘出後の残存腎における miRNA マイクロアレイ

Name	Fold change	*p* value	miRBase accession No.
rno-miR-340-5p	-76.3	4.6E-13	MIMAT0004650
rno-miR-1843a-5p	-37.3	7.9E-11	MIMAT0024847
rno-miR-450b-5p	-37.4	2.8E-11	MIMAT0035746
rno-miR-653-5p	-34.1	8.4E-13	MIMAT0012838
rno-miR-9-3p	-15.8	1.3E-02	MIMAT0004708
rno-miR-1843b-3p	-15.2	1.7E-02	MIMAT0035731
rno-miR-31-5p	-14.1	1.3E-02	MIMAT0000810
rno-miR-221-3p	-13.7	1.6E-02	MIMAT0000891
rno-miR-194-3p	-13.2	1.4E-02	MIMAT0017148

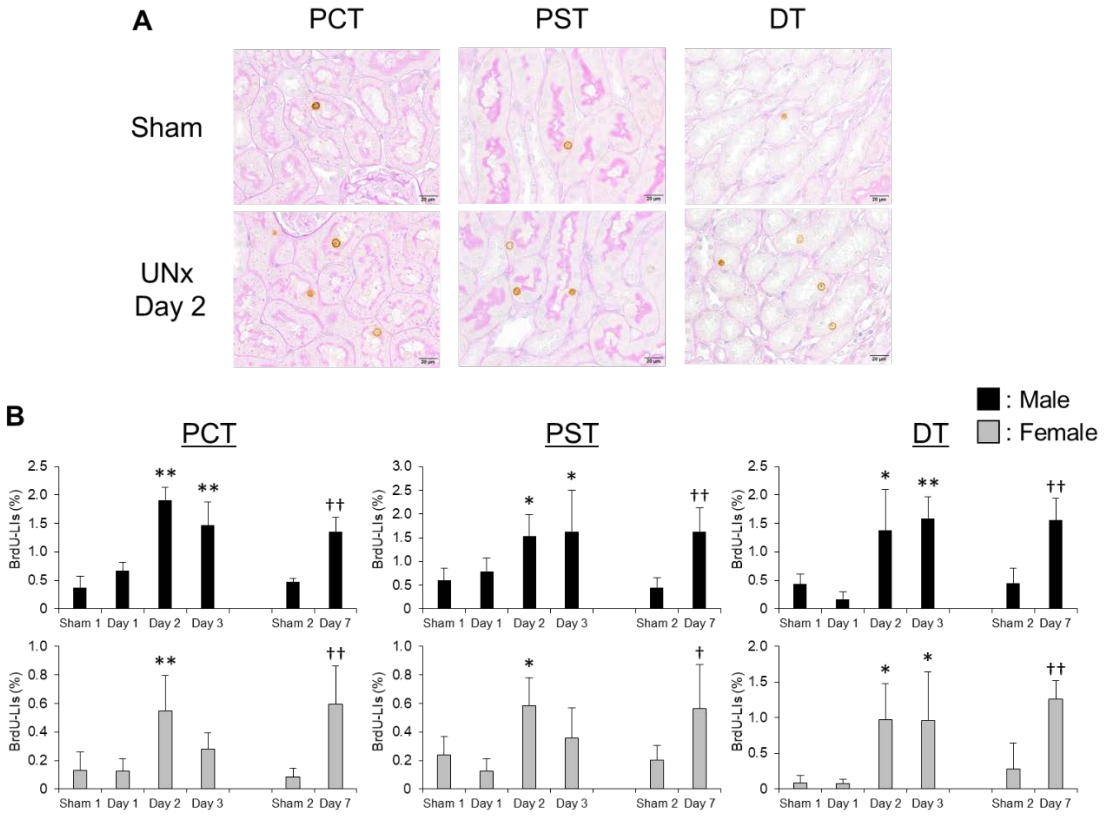

Figure 1. 片側腎摘出（UNx）後の残存腎の近位尿細管曲部（PCT），近位尿細管直部（PCT）および遠位尿細管（DT）における BrdU 免疫染色（A）および BrdU 陽性細胞率（B）.

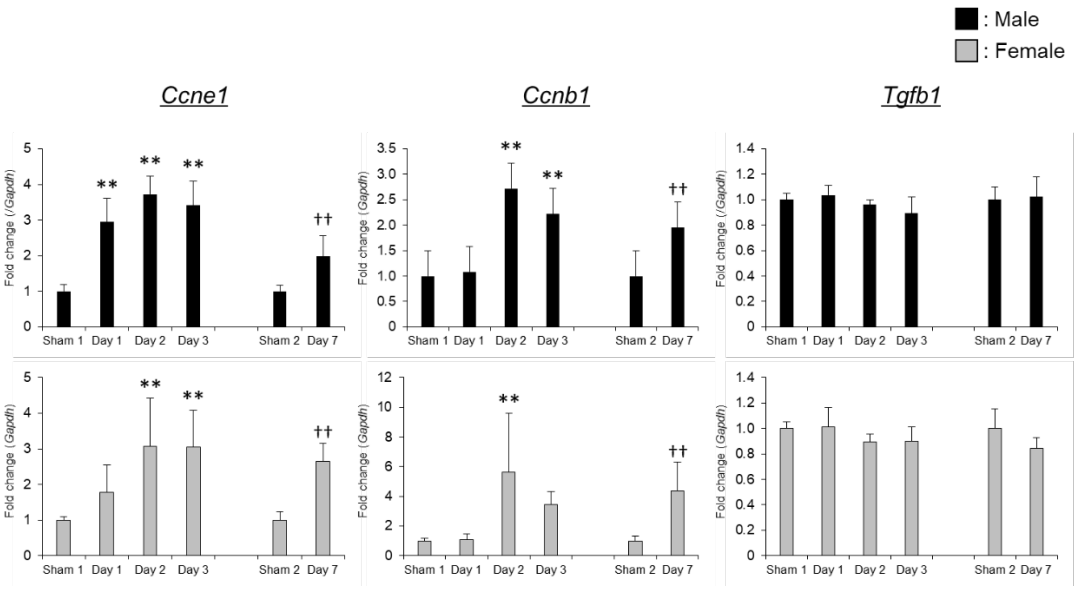

Figure 2. 片側腎摘出後の残存腎における Cyclin E1（*Ccne1*），Cyclin B1（*Ccnb1*）および TGF-β1（*Ggfb1*）の mRNA 発現解析.

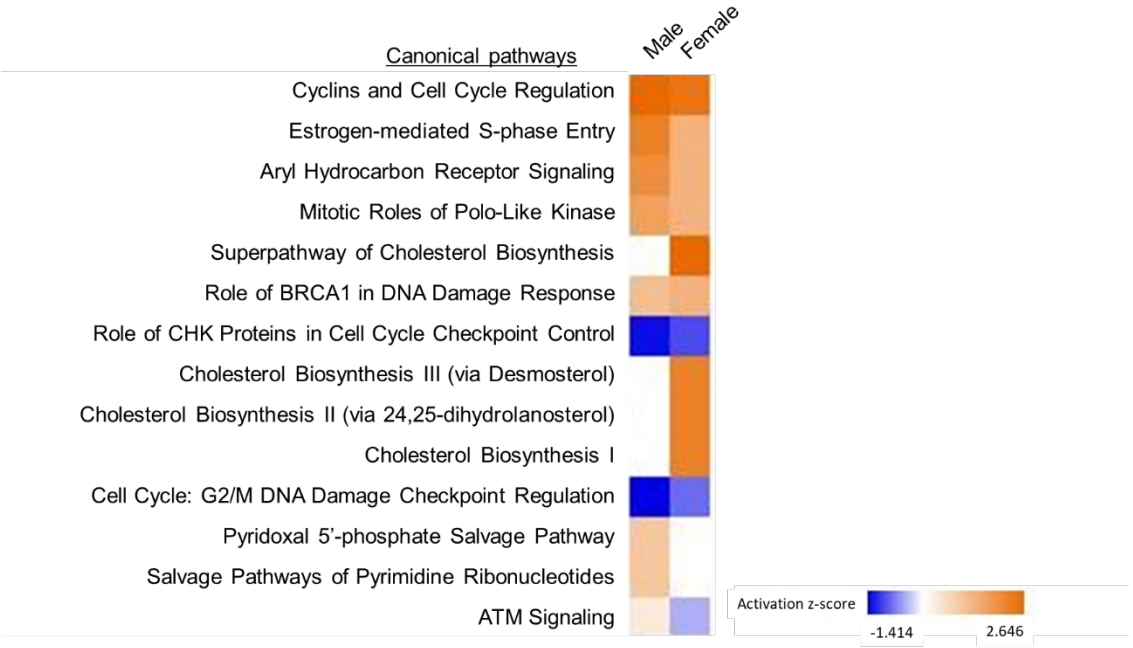

Figure 3. 片側腎摘出後の残存腎における mRNA マイクロアレイデータを用いた New Comparison Analysis.

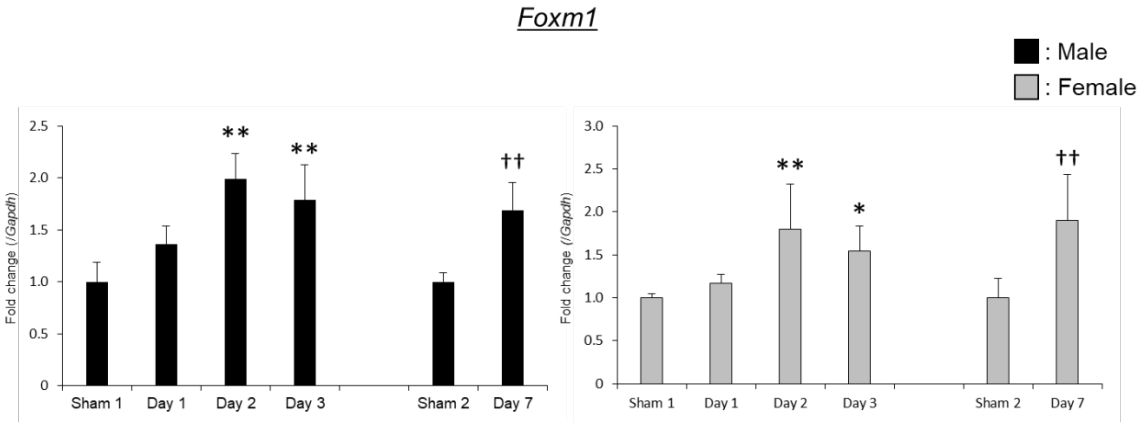

Figure 4. 片側腎摘出後の残存腎における FOXM1 の mRNA 発現解析.

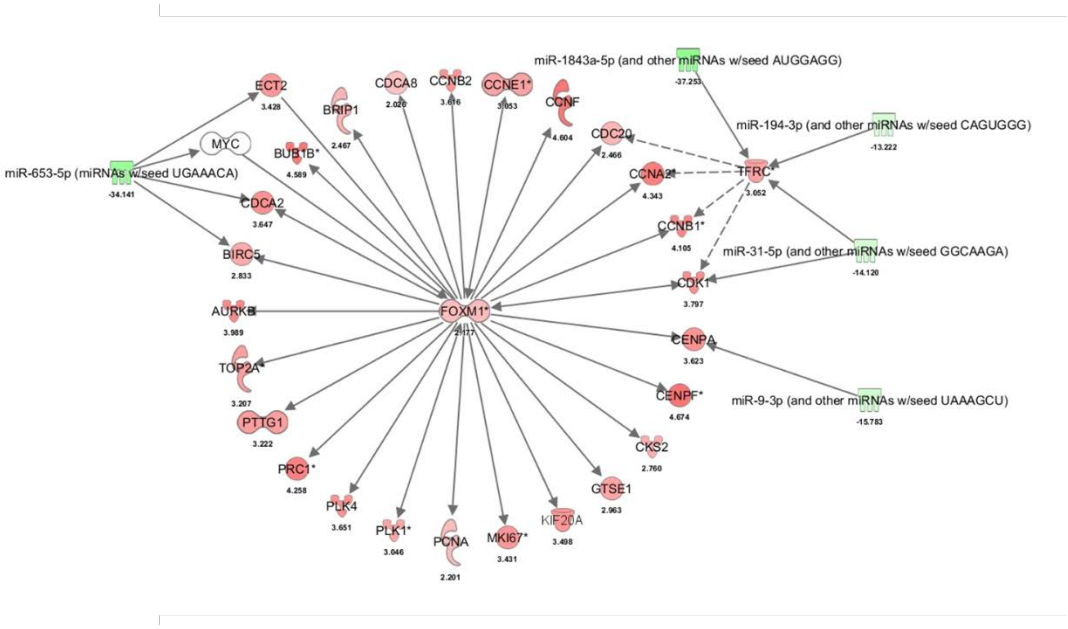

Figure 5. 片側腎摘出後の残存腎における mRNA-miRNA 統合解析

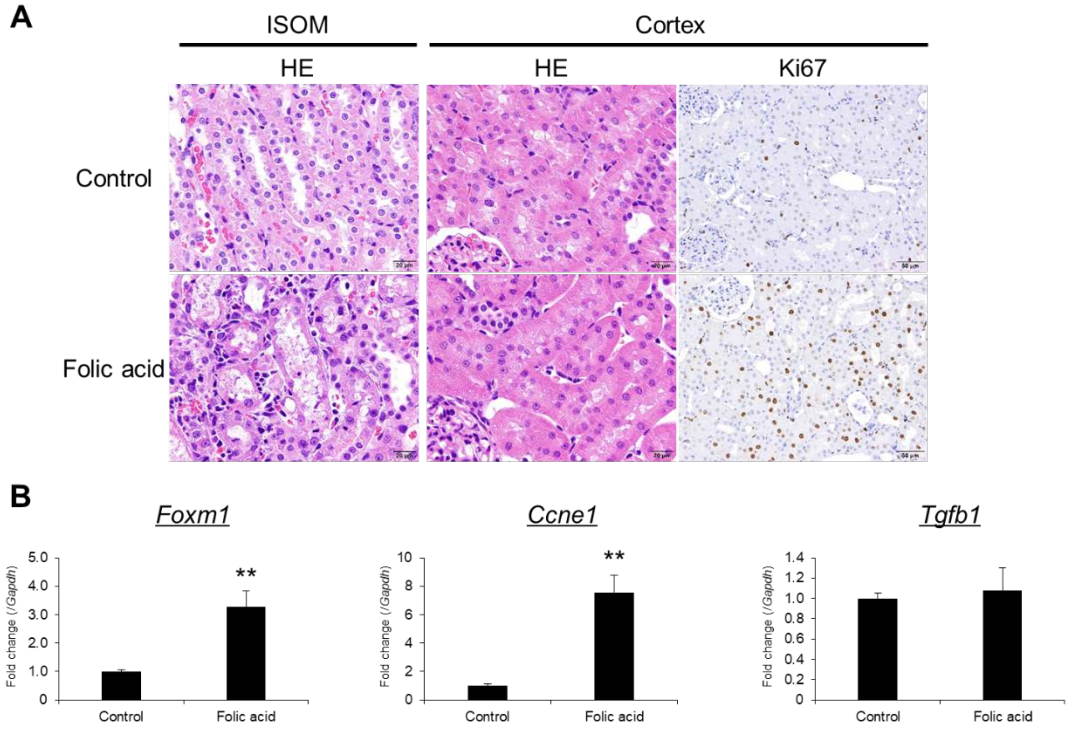

Figure 6. Folic acid 投与ラット腎臓における髄質外層内帯（ISOM）および皮質における病理組織学的解析および Ki67 免疫染色（A）ならびに皮質における FOXM1（*Foxm1*），Cyclin E1（*Ccne1*）および TGF-β1（*Ggfb1*）の mRNA 発現解析

厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）

OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

令和元年度 分担研究報告書

毒性等情報収集

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研究要旨

近年 OECD では、AOP に基づいて *in silico*、*in vitro*、*in vivo* の情報を組み合わせて化学物質の有害性を評価する Integrated Approaches to Testing and Assessment (IATA)および Defined Approach (DA)の開発が進められている。厚生労働科学研究化学物質リスク研究事業における化学物質の有害性評価の迅速化・高度化・標準化に関する研究および化学物質の新たなリスク評価手法の開発に関する研究の成果を、AOP の開発や代替試験法の公定化、IATA のコンセプトに基づいた化学物質のリスク評価の促進、規制・ガイドラインの新設や見直し等に反映させていくためには、当該研究事業で取得された新規有害性評価系のデータをとりまとめ、化学物質情報や毒性情報などとともに統合して利活用することが求められる。そこで本研究では、平成 30 年度に開始された厚生労働科学研究化学物質リスク研究事業公募型研究 4 課題について、構築する新規有害性評価系、試験物質とその試験データおよび AOP 開発、試験法公定化などの観点から整理することとし、今後の研究開発の参考情報とする。

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A. 研究目的

試験データのない数多くの化学物質の安全性評価が大きな課題となっている。さらに、動物福祉の観点から動物実験の削減の流れも着実に進んでいる。こうした動向に対応するため、近年OECDではAOPに基づいて*in silico*、*in vitro*、*in vivo*の情報を組み合わせて化学物質の安全性を評価する統合的アプローチ（Integrated Approaches to Testing and Assessment, IATA）のコンセプトの確立へ向けた試みが進められてきた。

IATAによる評価のワークフローは、現在以下のように整理されている（図1）。

① 課題を設定する。規制上のニーズ、制約、許容される不確実性を整理する。
② 関心のある化学物質について入手可能な既存の情報（*in vivo, in vitro, in silico*等）を収集し、Weight of Evidence (WoE)により統合的に解析し、規制上の結論を得る。
③ 既存の情報が不十分な場合は、効率的な試験戦略を立て、新規の情報を取得し、規制上の決定を下す。

IATAの実施において、AOPは種々の情報の

因果関係を明確化し、結論の導出に必要な情報の同定に役立つと期待されている。

さらにOECDでは、より透明性と客観性が高く標準化されたIATAを構築することを目的としてDefined Approach (DA)の検討が進められており、そのガイダンスを公開している。どのようなデータが必須であり、それぞれについてどのような重みづけを行うべきかが重要であり、それをサポートするのがAOPであると考えられている。

国際的には、IATAやDAの行政的な実運用へ向けて、種々のケーススタディを実施して、得られた経験・教訓を整理してガイダンスを開発する取り組みが進められている。今後は、動物実験への依存度を軽減しつつ、化学物質が発現しうるヒトへの毒性を効率的かつ高精度で予測するために、IATAやDAに基づいてヒト健康リスク評価のストラテジーを進化させる必要がある。

厚生労働科学研究化学物質リスク研究事業では、化学物質の有害性評価の迅速化・高度化・標準化に関する研究、化学物質の新たなリスク評価手法の開発に関する研究を推進し、成果をあげてきた。今後、これらの成果を、規制・ガイドラインの新設や見直し、さらには日常生活に利用される種々の化学物質のリスク評価等に反映させていくためには、当該研究事業で開発された新規有害性評価系のデータを、化学物質情報や関連物質の毒性情報などとともに統合して利活用することが求められる。そのためには、各研究課題の成果を整理してデータを集積するとともに、体系的にデータを解析し、不足する情報の同定や研究事業で開発された評価系の有効利用による規制判断の支援や評価・試験の戦略を立てることが望まれるところである。

そこで、本研究では、厚生労働科学研究化学物質リスク研究事業公募型研究でこれまでに得られた成果の基礎試験データを集積し、IATAのコンセプトに基づいた安全性評価・審議の支援、評価・試験戦略の策定に必要な情報収集の効率化やAOP開発に役立てることを目的とする。

B. 研究方法

B.1. 化学リスク研究事業総合報告書の調査

以下の平成30年度開始の厚生労働科学研究化学物質リスク研究事業［公募型］計4課題の30年度報告書を入手して、毒性エンドポイントと解析の対象化学物質、評価系の構築状況ならびに試験結果を精査してExcel形式で整理した。さらに、各研究課題の分担研究について、AOPの構築とテストガイドライン化へ向けた位置づけを整理し、俯瞰する図をPowerPoint形式でまとめた。(http://mhlw-grants.niph.go.jp/niph/search/NIFL00.do)。

1. 平成30年度　化学物質の動物個体レベルの免疫毒性データ集積とそれに基づくMulti-ImmunoTox assay（MITA）による予測性試験法の確立と国際標準化（H30-化学-一般-001）
2. 平成30年度　家庭用品化学物質が周産期の中枢神経系に及ぼす遅発性毒性の評価系作出に資する研究（H30-化学-一般-003）
3. 平成30年度　生体影響予測を基盤としたナノマテリアルの統合的健康影響評価方法の提案（H30-化学-一般-004）
4. 平成30年度　血液中の核酸をバイオマーカーに用いた化学物質の高感度な有害性評価に資する研究（H30-化学-一般-002）

C. 研究結果および考察

C1. 各研究事業における新規有害性評価系の開発状況と AOP 開発へ向けた課題

各研究事業の分担研究ごとに、以下の項目 1）各分担研究課題、2）目的、3）研究対象物質、4）材料と方法、5）結論、を設定し情報を整理した（4 研究課題、24 分担研究）。表 1 に一例を示す。さらにそれらを総合して AOP 開発へ向けた位置づけを整理し、課題を考察した。

“化学物質の動物個体レベルの免疫毒性データ集積とそれに基づく Multi-ImmunoTox assay（MITA）による予測性試験法の確立と国際標準化”においては、まず、化学物質の免疫毒性データの作成を国際協力により進めている。25 物質のデータ集積を行い、それらを *in vivo*, *ex vivo*、*in vitro* データに分類した。その結果、各化学物質の大凡の免疫毒性 profile が俯瞰可能となった。MITA は、T 細胞および単球のサイトカイン転写調節に及ぼす化学物質の影響をレポーター遺伝子の発光を利用して評価する *in vitro* 免疫毒性評価試験法である。そのうち IL-2 Luc アッセイについては、プロトコールならびにクライテリアの改訂によるバリデーション研究の再評価が進んでいる。さらに、転写誘導抑制を指標とした IL-1 Luc assay については、Phase 0 で国際バリデーション実行委員会にて選定した 3 物質について、3 施設間で再現性を確認し、Phase I で同委員会にて選定した 5 化学物質をコード化し施設内再現性を検証している。なお、免疫毒性の AOP 開発は、“OECD プログラムにおいて TG と DA を開発するための AOP に関する研究”で実施中である。

“家庭用品化学物質が周産期の中枢神経系に及ぼす遅発性毒性の評価系作出に資する研究”では、家庭用品に含まれる化学物質について、妊婦（胎児）や小児をシグナル異常に脆弱な集団と位置づけ、生活環境レベルでの低用量暴露による遅発性の中枢神経系への影響を検討している。平成 30 年度は、(1) 発生発達期にかけてのペルメトリンの低用量長期飲水投与による成熟後の中枢神経系への影響解析と、(2) 発生発達期にかけての塩化トリブチルスズの低用量長期飲水投与による成熟後の中枢神経系への影響解析を実施している。図 2(A)には、本研究事業の各分担研究の役割分担を俯瞰する図をまとめた。各分担研究で対象とする被験物質を共通化し、遺伝子発現プロファイル、DNA メチル化影響など分子レベルでの解析および脳神経回路イメージング解析、神経幹細胞動態解析などの神経科学的解析により、低用量化学物質の周産期暴露による成熟後マウスの行動様式への影響の機序について体系的に解明を進めており、遅発性毒性の評価系構築を進めている。OECD では近年新しい *in vivo* 毒性試験の公定化は積極的ではないとされるが、*in vitro* 試験への代替が困難な遅発性毒性評価系について、国際的なガイドライン作出へ向けた情報発信を行っている。

“生体影響予測を基盤としたナノマテリアルの統合的健康影響評価方法の提案”では、(1) ナノマテリアルの *in vitro* 安全性評価法の高度化、(2) AOP の確立、(3) 毒性試験データベースの作成、(4) *in silico* 生体影響予測を組合せたナノマテリアルの統合的健康影響評価方法の構築を目指

している。図 2(B)には、本研究事業の各分担研究の役割分担を俯瞰する図をまとめた。ナノマテリアルは種類が多いため、それらを用いた多面的な分担研究が進められている。しかし、AOP の開発および *in silico* 手法を組合せた統合的評価方法の確立を加速化するためには、他の研究事業のように、被験物質の共通化、毒性エンドポイントの絞り込み、*in vivo* 毒性との関連付けが課題となる可能性がある。

“血液中の核酸をバイオマーカーに用いた化学物質の高感度な有害性評価に資する研究”では、化学物質ばく露後のマウスの血液中の核酸のうち、エクソソーム RNA の網羅的解析により、標的臓器を特定し、更に毒性発現機序の解明を目指すことで、化学物質の「次世代型」有害性評価による迅速化、高度化および標準化を行うことを目的としている。新規の研究事業であり、平成 30 年度は、マウス血液からのエクソソーム RNA 単離の標準化を行った。さらに、化学構造の基本構造は同じであるが、側鎖の違いなどによりその毒性の強さや発現する臓器に違いがあるベンゾトリアゾール類 5 物質について、次世代シーケンス解析を実施した。本研究は基礎研究の段階であるが、将来、迅速な有害性評価系としての利活用、AOP 構築を検討するとなれば、分子マーカーと *in vivo* の臓器毒性の因果関係を明確化することが課題のひとつとして想定される。

各研究のAOP構築へ向けた共通の課題として、分子開始イベント（Molecular Initiating Event, MIE）情報の不足が挙げられる。MIE は化学物質と生体分子との相互作用により、毒性発現に至る最初の引き金となる反応である。OECDでのAOP開発においては必須の情報であり、例えば”Histone deacetylase inhibition leading to testicular toxicity” (AOP212)のように、MIEに関する情報はAOPのタイトルに含められる。AOP開発の促進のためには、化学物質の毒性発現に寄与する標的分子を効率的に同定する研究手法の開発が求められる。

AI-based Chemical Safety Assessment Forward Evolution platform (AI-CSAFE)は、国衛研が長年にわたって整備してきた信頼性の高い毒性試験データを統合したビッグデータベースと、医薬品・食品・化学物質3分野にまたがるレギュラトリーサイエンスに基づく安全性評価の専門的知見並びに高精度の安全性研究の経験とをAIを活用し統合させることを目指した、現在開発中の安全性予測支援プラットフォームである。その共通基盤としてデータベースの充実に注力している。リスク評価書やその引用文献に加えて、AOP-Wikiに登録されているKey Eventに関連した文献情報をAI-CSAFEの情報収集源として追加することを検討する。

D. 結論

毒性等情報収集では、厚生労働科学研究化学物質リスク研究事業（公募型）で実施された成果を、IATAのコンセプトに基づいた安全性評価やその基盤となるAOP開発に役立てるため、年次報告書を精査した。各研究課題の分担研究について、特にAOP開発へ向けた位置づけを整理し、課題を考察した。

E. 研究発表

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G. 知的財産権の出願・登録状況

該当なし

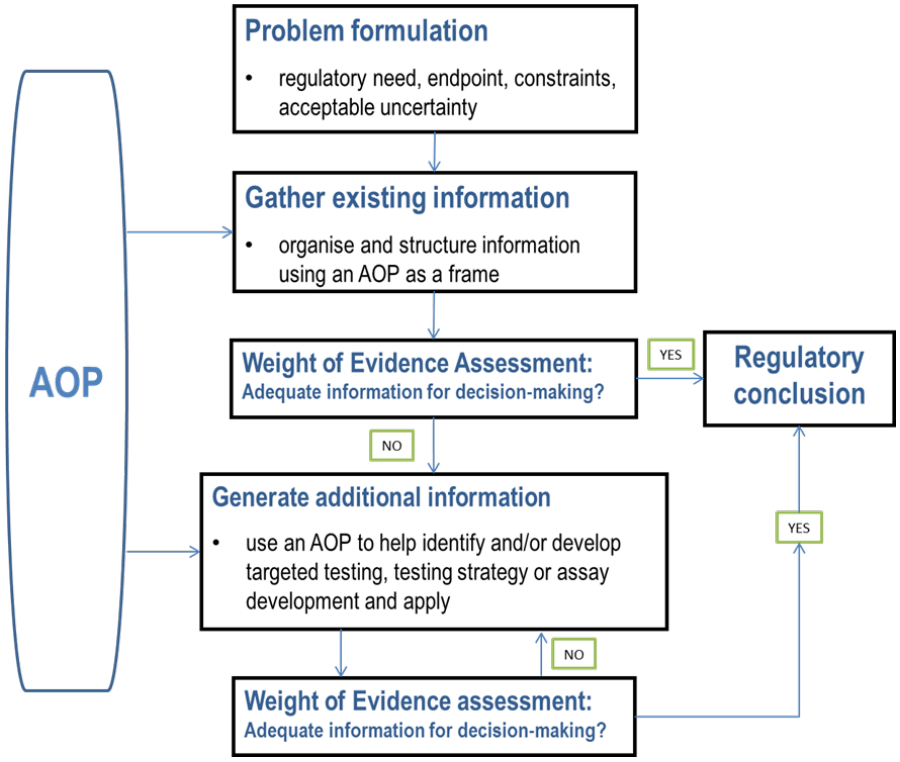
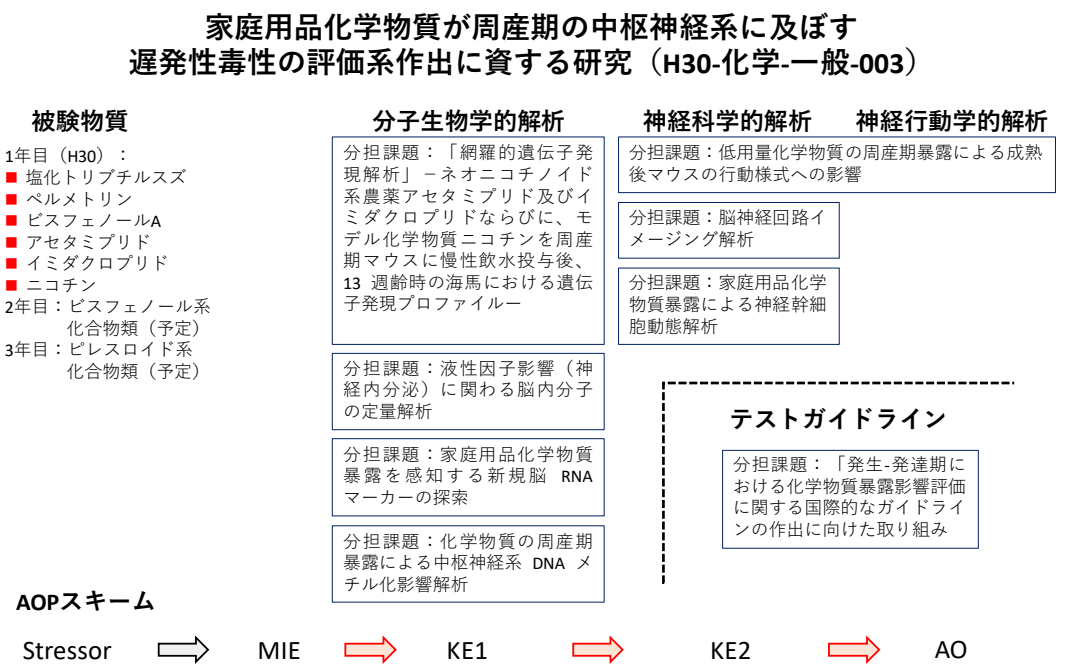


図 1　OECD が提唱する規制判断のための IATA のワークフロー

表 1　各研究事業の分担研究の情報整理の例

研究課題	化学物質の動物個体レベルの免疫毒性データ集積とそれに基づく Multi-ImmunoTox assay（MITA）による予測性試験法の確立と国際標準化（H30-化学-一般-001）
分担研究課題	免疫毒性評価試験法（Multi-ImmunoTox assay）国際標準化へ向けた評価法の検討
目的	MITA 試験法確立と OECD ガイドライン化：TGCHAC-4A 細胞を用いた試験法の確立を目指し、バリデーションの技術移転性の確認（Phase0）および Phase1 試験の実施
研究対象物質	Phase 0 ：Dapsone，Diethanolamine，p-Nitroaniline（国際バリデーション実行委員会にて選定） Phase I ：1 セット 5 種類のコード化した被験物質 3 セット（同委員会にて選定）
材料	IL-1β と内部標準としての G3PDH プロモーターに SLG および SLR ルシフェラーゼ遺伝子をそれぞれ繋いだ人工染色体発現ベクターを THP-1 細胞に導入した 2 色発光細胞株 TGCHAC-4A（THP-G1β）
方法	免疫毒性試験法における細胞培養、被験物質調製及び添加、及びルシフェラーゼアッセイ、試験結果の判定基準等については Multi-Immuno Tox Assay protocol 案 Ver.007E（Phase0）および Ver.008E（Phase1）に準ずる。発光の計測には多検体発光測定装置 Phelios を用いた。
結果	Phase 0：バリデーション試験の実施 3 施設（産総研健康工学研究部門、産総研バイオメディカル研究部門、東北大医学部皮膚科）の結果を比較検討した結果、良好な施設内、施設間再現性が得られ、技術移転性を確認できた。 Phase I ：5 物質全てにおいて、3 セットで同一の判定結果となり、良好な施設内再現性を確認した。一方、各セットにおける 1st~4th experience を確認すると、3 つの被験試薬（MIB503、MIB601、MIB602）において、N; No effect と S; Suppression の両判定があった。
結論	プロトコールの改善や判定基準の再検証を進めることにより、より正確性、再現性を向上した試験法の確立が見込まれる。

(A)



(B)

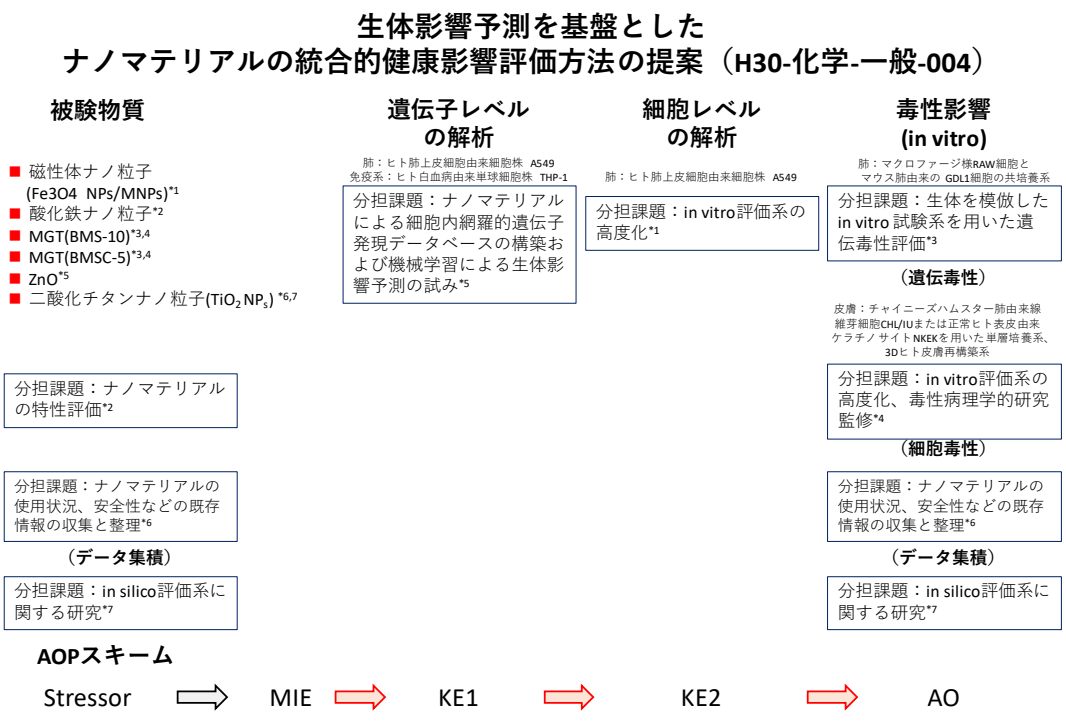


図 2　各研究事業の構成と AOP スキームとの関係

別紙５

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
Kojima H, Sakai Y, Tanaka N	Japanese Contributions to the Development of Alternative Test Methods	Michael Balls, Robert Combes and Andrew Worth	The History of Alternative Test Methods in Toxicology	Elsevier	Netherlands	2019	79-85

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kobayashi-Tsukumo H, Oiji K, Xie D, Sawada Y, Yamashita K, Ogata S, Kojima H, Itagaki H	Eliminating the contribution of lipopolysaccharide to protein allergenicity in the human cell-line activation test (h-CLAT)	J Toxicol Sci.	44(4)	283-297	2019
荻原 琢男, 細野 麻友, 小島 肇	ヒト肝細胞の3次元培養スフェロイドモデルの新展開	日本薬理学雑誌	153(5)	235-241	2019
Fujita M, Yamamoto Y, Watanabe S, Sugawara T, Wakabayashi K, Tahara Y, Horie N, Fujimoto K, Kusakari K, Kurokawa Y, Kawakami T, Kojima K, Sozu T, Nakayama T, Kusao T, Richmond J, Nicole K, Kim BH, Kojima H, Kasahara T, Ono A	The within- and between-laboratory reproducibility and predictive capacity of the in chemico amino acid derivative reactivity assay: Results of validation study implemented in four participating laboratories	J Appl Toxicol.	39(11)	1492-1505	2019
Mizoi K, Hosono M, Kojima H, Ogihara T	Establishment of a primary human hepatocyte spheroid system for evaluating metabolic toxicity using dacarbazine under conditions of CYP1A2 induction	Drug Metab Pharmacokinet	35	201-206	2020

Akimoto M, Yamamoto Y, Watanabe S, Yamaga H, Yoshida K, Wakabayashi K, Tahara Y, Horie N, Fujimoto K, Kusakari K, Kamiya K, Kojima K, Kawakami T, Kojima H, Ono A, Kasahara T, Fujita M	Oxidation of a cysteine-derived nucleophilic reagent by dimethyl sulfoxide in the amino acid derivative reactivity assay	J Appl Toxicol	40(6)	843-854	2020
Marx U, Akabane T, Andersson TB, Baker E, Beilmann M, Beken S, Brendler-Schwaab S, Cirit M, David R, Dehne EM, Durieux I, Ewart L, Fitzpatrick SC, Frey O, Fuchs F, Griffith LG, Hamilton GA, Hartung T, Hoeng J, Hogberg H, Hughes DJ, Ingber DE, Iskandar A, Kanamori T, Kojima H, Kuehnl J, Leist M, Li B, Loskill P, Mendrick DL, Neumann T, Pallocca G, Rusyn I, Smirnova L, Steger-Hartmann T, Tagle DA, Tonevitsky A, Tsyb S, Trapecar M, Van de Water B, Van den Eijnden-van Raaij J, Vulto P, Watanabe K, Wolf A, Zhou X, Roth A	Biology-inspired microphysiological systems to advance patient benefit and animal welfare in drug development	ALTEX.	Online ahead of print.	doi: 10.14573/altex.2001241	2020

Kimura Y, Yasuno R, Watanabe M, Kobayashi M, Iwaki T, Fujimura C, Ohmiya Y, Yamakage K, Nakajima Y, Kobayashi M, Mashimo N, Takagi Y, Omori T, Corsini E, Germolec D, Inoue T, Rogen EL, Kojima H, Aiba S	An international validation study of the IL-2 Luc assay for evaluating the potential immunotoxic effects of chemicals on T cells and a proposal for reference data for immunotoxic chemicals	Toxicol In Vitro	66	doi: 10.1016/j.tiv.2020.104832	2020
Hidaka T, Fujimura T, Aiba S	Aryl hydrocarbon receptor modulates carcinogenesis and maintenance of skin cancers	Frot Med	6	doi: 10.3389/fmed.2019.00194	2019
Iyama Y, Sato H, Seto Y, Onoue S	A new photosafety screening strategy based on in chemico photoreactivity and in vitro skin exposure for dermally-applied chemicals	Toxicology Letters	317	45-52	2019
Seto Y, Ueno K, Suzuki H, Sato H, Onoue S	Development of novel lutein nanocrystal formulation with improved oral bioavailability and ocular distribution	Journal of Functional Foods	61	https://doi.org/10.1016/j.jff.2019.103499	2019
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Yamada S, Kuraoka S, Ito Y, Kato Y, Onoue S	Muscarinic receptor binding of fesoterodine, 5-hydroxymethyl tolterodine, and tolterodine in rat tissues after the oral, intravenous, or intravesical administration	Journal of Pharmacological Sciences	140(1)	73-78	2019
Yamada T, Kumai Y, Kodama H, Nishimoto K, Miyamaru S, Onoue S, Orita Y	Effect of pirfenidone injection on ferret vocal fold scars: A preliminary in vivo study	Laryngoscope	130(3)	726-731	2020

Uchida A, Ohtake H, Suzuki Y, Sato H, Seto Y, Onoue S, Oguchi T	Photochemically stabilized formulation of dacarbazine with reduced production of algogenic photodegradants	International Journal of Pharmaceutics	564(10)	492-8	2019
Iyama Y, Sato H, Seto Y, Onoue S	Photochemical and pharmacokinetic characterization of orally administered chemicals to evaluate phototoxic risk	Journal of Pharmaceutical Sciences	108(3)	1303-8	2019
Nagayasu M, Ozeki K, Sakurai Y, Tsutsui H, Onoue S	Simplified method to determine the efflux ratio on P-glycoprotein substrates using three-compartment model analysis for Caco-2 cell assay data	Pharmaceutical Research	37(1)	doi: 10.1007/s11095-019-2729-x.	2019
Halder S, Suzuki H, Seto Y, Sato H, Onoue S	Megestrol acetate-loaded self-micellizing solid dispersion system for improved oral absorption and reduced food effect	Journal of Drug Delivery Science and Technology	49	586-93	2019
Saito S, Osamura T, Kikuoka H, Tanino T, Onoue S	BIND, a novel analytical approach for monitoring powder adhesion at the die wall with use of the surface replication method	International Journal of Pharmaceutics	567	doi: 10.1016/j.ijpharm.2019.118467.	2019
Sato H, Kaneko Y, Yamada K, Ristroph KD, Lu HD, Seto Y, Chan HK, Prud'homme RK, Onoue S	Polymeric Nanocarriers With Mucus-Diffusive and Mucus-Adhesive Properties to Control Pharmacokinetic Behavior of Orally Dosed Cyclosporine A	Journal of Pharmaceutical Sciences	190(2)	1079-1085	2020
Iyama Y, Sato H, Seto Y, Onoue S	Strategic photosafety screening system consisting of in chemico photoreactivity and in vitro skin exposure for quinolone derivatives	European Journal of Pharmaceutical Sciences	146	105257	2020

Seto Y, Ohtake H, Sato H, Onoue S	Phototoxic risk assessment of dermally-applied chemicals with structural variety based on photoreactivity and skin deposition	Regulatory Toxicology and Pharmacology	113	doi: 10.1016/j.yrtph.2020.104619.	2020
Sugiyama K, Furusawa H, Honma M	Detection of epigenetic effects of citrinin using a yeast-based bioassay	Mycotoxin Res.	35	363-36	2019
Matsushita K, Toyda T, Yamada T, Morikawa T, Ogawa K	Comprehensive expression analysis of mRNA and microRNA for investigation of compensatory mechanisms in the rat kidney after unilateral nephrectomy	Journal of Applied Toxicology	Online ahead of print	doi: 10.1002/jat.3990	2020
Jojima K, Yamada T, Hirose A	Development of a hepatotoxicity prediction model using in vitro assay data of key molecular events	Fundam. Toxicol. Sci.	6	327-32	2019
Inoue K, Suzuki H, Yamada T	Comprehensive toxicity evaluation of cyclopentyl methyl ether (CPME) for establishing a permitted daily exposure level	Fundam Toxicol Sci.	6	145-165	2019
Yamada T, Matsumoto M, Miura M, Hirose A	Case study on the use of integrated approaches to testing and assessment for testicular toxicity of ethylene glycol methyl ether (EGME)-related chemicals	Organisation for Economic Co-operation and Development (OECD), Series on Testing & Assessment	308	1-75	2019

Patlewicz G, Lizarraga LE, Rua D, Allen DG, Daniel AB, Fitzpatrick SC, Garcia-Reyero N, Gordon J, Hakkinen P, Howard AS, Karmaus A, Matheson J, Mumtaz M, Richarz A, Ruiz P, Scarano L, Yamada T, Kleinstreuer N	Exploring current read-across applications and needs among selected U.S. Federal Agencies	Regul. Toxicol. Pharmacol.	106	197-209	2019
Tachibana K, Kass GEN, Ono A, Yamada T, Tong W, Doerge DR, Yamazoe Y	A Summary Report of FSCJ Workshop "Future Challenges and Opportunities in Developing Methodologies for Improved Human Risk Assessments"	Food Safety	7	83-89	2019
山田隆志，足利太可雄，小島肇，広瀬明彦	AOP（Adverse Outcome Pathway；有害性発現経路）に基づいた化学物質の安全性評価へ向けたチャレンジ	YAKUGAKU ZASSHI	140	481-484	2020
田邊思帆里，広瀬明彦，Maurice Whelan，山田隆志	遺伝子ネットワーク解析による分子パスウェイ解明及びAOP開発状況について	YAKUGAKU ZASSHI	140	485-489	2020

令和２年３月２７日

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機関名　国立医薬[redacted]

所属研究機関長　職　名　所長

氏　名　奥田　晴[redacted]

次の職員の令和元年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　化学物質リスク研究事業

２．研究課題名　OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

３．研究者名　（所属部局・職名）　安全性予測評価部　第二室　室長

（氏名・フリガナ）　小島　肇（コジマ　ハジメ）

４．倫理審査の状況

	該当性の有無	左記で該当がある場合のみ記入（※1）		
	有　無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□　☑	□		□
遺伝子治療等臨床研究に関する指針	□　☑	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□　☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□　☑	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　　）	□　☑	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

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５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □（無の場合はその理由：　　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □（無の場合は委託先機関：　　）
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当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑（有の場合はその内容：　　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和２年３月２７日

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機関名　国立医薬品[redacted]

所属研究機関長　職　名　所長

氏　名　奥田　晴宏[redacted]

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３．研究者名　（所属部局・職名）　安全性生物試験研究センター病理部　部長

（氏名・フリガナ）　小川　久美子　（オガワ　クミコ）

４．倫理審査の状況

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	有	無	審査済み	審査した機関	未審査（※2）
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遺伝子治療等臨床研究に関する指針	□	☑	□		□
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厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	☑	□	☑	国立医薬品食品衛生研究所	□
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３．研究者名　（所属部局・職名）　安全性生物試験研究センター病理部　客員研究員

（氏名・フリガナ）　西川　秋佳（ニシカワ　アキヨシ）

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（氏名・フリガナ）　チョウ　ヨンマン（チョウ　ヨンマン）

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（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和２年　３月１８日

厚生労働大臣　殿

機関名　国立大学法[redacted]

所属研究機関長　職　名　　総長

氏　名　大野　英[redacted]

次の職員の令和元年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　化学物質リスク研究事業

２．研究課題名　OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

３．研究者名　（所属部局・職名）　大学院医学系研究科　・教授

（氏名・フリガナ）　相場　節也　（アイバ　セツヤ）

４．倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入（※1）		
	有	無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□	■	□		□
遺伝子治療等臨床研究に関する指針	□	■	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□	■	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□	■	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　　）	□	■	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。
（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ■　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ■　無 □（無の場合はその理由：　　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ■　無 □（無の場合は委託先機関：　　）
当研究に係るＣＯＩについての報告・審査の有無	有 ■　無 □（無の場合はその理由：　　）
当研究に係るＣＯＩについての指導・管理の有無	有 ■　無 □（有の場合はその内容：研究実施の際の留意点を示した　　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和２年４月14日

厚生労働大臣　殿

機関名　神奈川県衛生研究所

所属研究機関長　職　名　神奈川県衛生研究[redacted]

氏　名　髙崎　智彦

次の職員の令和元年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　化学物質リスク研究事業

２．研究課題名　OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

３．研究者名　（所属部局・職名）　神奈川県衛生研究所　理化学部・　主任研究員

（氏名・フリガナ）　大森（オオモリ）　清美（キヨミ）

４．倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入（※1）		
	有	無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□	■	□		□
遺伝子治療等臨床研究に関する指針	□	■	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□	■	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□	■	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　）	□	■	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。
（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ■　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ■　無 □（無の場合はその理由：　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ■　無 □（無の場合は委託先機関：　）
当研究に係るＣＯＩについての報告・審査の有無	有 ■　無 □（無の場合はその理由：　）
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ■（有の場合はその内容：　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

年　月　日

厚生労働大臣　殿

機関名　静岡県立大学

所属研究機関長　職　名　学長

氏　名　鬼頭 宏 [redacted]

次の職員の令和元年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

1．研究事業名　化学物質リスク研究事業

2．研究課題名　OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

3．研究者名　（所属部局・職名）　静岡県立大学 薬学部 教授

（氏名・フリガナ）　尾上 誠良 （オノウエ サトミ）

4．倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
ヒトゲノム・遺伝子解析研究に関する倫理指針	□	☑	□		□
遺伝子治療等臨床研究に関する指針	□	☑	□		□
人を対象とする医学系研究に関する倫理指針 (※3)	□	☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	☑	□	☑	静岡県立大学動物実験委員会	□
その他、該当する倫理指針があれば記入すること（指針の名称：　）	□	☑	□		□

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

(※2) 未審査に場合は、その理由を記載すること。
(※3) 廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

5．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

6．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □(無の場合はその理由：　)
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □(無の場合は委託先機関：　)
当研究に係るＣＯＩについての報告・審査の有無	有 □　無 ☑ (無の場合はその理由：　自己申告が基準以下のため　)
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑ (有の場合はその内容：　)

(留意事項)　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和２年３月２７日

厚生労働大臣　殿

機関名　国立医薬品[redacted]

所属研究機関長　職　名　所長

氏　名　奥田　晴宏

次の職員の令和元年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　化学物質リスク研究事業

２．研究課題名　OECD プログラムにおいて TG と DA を開発するための AOP に関する研究

３．研究者名　（所属部局・職名）　安全性予測評価部　第二室　主任研究官

（氏名・フリガナ）　足利 太可雄（アシカガ　タカオ）

４．倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入（※1）		
	有	無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□	☑	□		□
遺伝子治療等臨床研究に関する指針	□	☑	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□	☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□	☑	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　）	□	☑	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。
（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □（無の場合はその理由：　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □（無の場合は委託先機関：　）
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □（無の場合はその理由：　）
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑（有の場合はその内容：　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和２年３月２７日

厚生労働大臣 殿

機関名　国立医薬[redacted]

所属研究機関長　職　名　所長

氏　名　奥田　晴[redacted]

次の職員の令和元年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　化学物質リスク研究事業

２．研究課題名　OECDプログラムにおいてTGとDAを開発するためのAOPに関する研究

３．研究者名　（所属部局・職名）　安全性生物試験研究センター 変異遺伝部・室長

（氏名・フリガナ）　杉山圭一・スギヤマケイイチ

４．倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入（※1）		
	有	無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□	☑	□		□
遺伝子治療等臨床研究に関する指針	□	☑	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□	☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□	☑	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　）	□	☑	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）　特になし。

（※2）未審査に場合は、その理由を記載すること。
（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □（無の場合はその理由：　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □（無の場合は委託先機関：　）
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □（無の場合はその理由：　）
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑（有の場合はその内容：　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和２年３月２７日

厚生労働大臣 殿

機関名　国立医薬品[redacted]

所属研究機関長　職　名　所長

氏　名　奥田　晴宏[redacted]

次の職員の令和元年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　化学物質リスク研究事業

２．研究課題名　OECDプログラムにおいてTGとDAを開発するためのAOPに関する研究

３．研究者名　（所属部局・職名）　安全性生物試験研究センター病理部・主任研究官

（氏名・フリガナ）　松下　幸平（マツシタ　コウヘイ）

４．倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入（※1）		
	有	無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□	☑	□		□
遺伝子治療等臨床研究に関する指針	□	☑	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□	☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	☑	□	☑	国立医薬品食品衛生研究所	□
その他、該当する倫理指針があれば記入すること（指針の名称：　　）	□	☑	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。
（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □（無の場合はその理由：　　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □（無の場合は委託先機関：　　）
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □（無の場合はその理由：　　）
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑（有の場合はその内容：　　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和２年３月２７日

厚生労働大臣 殿

機関名　国立医薬品[redacted]

所属研究機関長　職　名　所長

氏　名　奥田　晴宏[redacted]

次の職員の令和元年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　化学物質リスク研究事業

２．研究課題名　OECDプログラムにおいてTGとDAを開発するためのAOPに関する研究

３．研究者名　（所属部局・職名）　安全性予測評価部　第四室　室長

（氏名・フリガナ）　山田　隆志（ヤマダ　タカシ）

４．倫理審査の状況

	該当性の有無	左記で該当がある場合のみ記入（※1）		
	有　無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□　☑	□		□
遺伝子治療等臨床研究に関する指針	□　☑	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□　☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□　☑	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　　）	□　☑	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。

（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □（無の場合はその理由：　　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □（無の場合は委託先機関：　　）
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □（無の場合はその理由：　　）
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑（有の場合はその内容：　　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。