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)

平成30年度　総括・分担研究報告書

研究代表者　小島　肇

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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 情報を活用する戦略がある。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 に関する TG 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 OECD の TG や AOP が採択されたものはなかった。ただし、来年度に 3 試験法の TG、1 件の AOP を成立できる目途がたった。

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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。特に、非遺伝毒性発がんは、催乳ホルモン（prolactin）や甲状腺刺激ホルモン（TSH）、甲状腺ホルモン（T4）を含む各種ホルモンレベルの増加と関連しているものも多く、それらは発がん性の KE (Key Event)となり得る。そこで、通常の反復投与毒性試験のプロトコルで採取される下垂体、甲状腺などを用いて prolactin、TSH、T4 などの発現量の増減を免疫組織化学染色法によって半定量する評価方法の確立を目指す。さらに、腎毒性の作用機構の開発も行う。これらや既存の 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、形質転換試験 Bhas42 法及びラット肝中期発がん性試験については、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、OECD での非遺伝毒性発がん IATA 及び皮膚感作性 DA の TG 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（小島）

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、分担及び協力研究者を支援した。

AOPマニュアルを作成するため、EAGMSTの資料を再調査した。

B.2. TG 開発

B.2.1. TG の採択に向けた動向（小島、尾上）

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、電話会議や専門家会議にて交渉した。

B.2.2. 免疫毒性 TG の提案（小島、相場）

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 会議を企画した。また、*in vitro* 免疫毒性試験に関する Detailed Review Paper(DRP)の SPSF を作成し、OECD に提案した。

B.2.3. 発がん性 TG の提案（小川）

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の TG 申請を行うための資料を整理し、SPSF を OECD に提案した。

B.2.4. Bhas42 細胞形質転換試験法の TG 開発（大森）

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試験法（OECD ガイダンスドキュメント No.231）である。OECD NGTxC IATA の専門家会議において、Bhas42CTA における非遺伝毒性発がん性発現の機序解析データを提供した。

B.3. AOPの開発

B.3.1. 免疫毒性のAOP開発（小島、足利、相場、久田）

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。

1) 文献調査の結果に基づいて、カルシニューリン阻害を Molecular initiating event（MIE）とし、T 細胞依存性抗体産生抑制（TDAR）を Adverse outcome（AO）とする AOP154 案 Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response を作成した。
2) 新たに免疫毒性 3 件の AOP を開発した。AOP の作成対象を決定し、文献調査に基づいて、MIE、AO 及びその間に介在する KE を定めて、SPSF（Standard Project Submission Form）を OECD に提案した。
3) IL-2 を分子初動因子とした AOP 開発を OECD に申請済であり、その作成を継続し、来年度の成立を目指した。

B.3.2. 発がん性 AOP の開発（西川、小川、久田）

1) 研究分担者の西川及び小川は、細胞毒性を初動因子とした鼻腔発がんの AOP 開発を OECD に申請済であり、細胞傷害と腫瘍発生との関連に係るデータ解析を進め、AOP 作成を継続し、再来年度の成立を目指した
2) 日本製薬工業会グループ（久田茂ほか）の協力を得て、ラットにおける非遺伝毒性発がん性 AOP の作成を開始し、SPSF を OECD に提案した。

B.3.3 光安全性の AOP 開発（尾上）

光化学的特性に焦点を当てた光毒性に関する AOP 案を作成した。

B.4. IATA 及び DA の開発

B.4.1. IATA の開発協力（西川、小川、大森）

OECD では、非遺伝毒性発がん性（NGTxC）検出を目的とした IATA の開発が行われており、この専門委員会のメンバーとして IATA の開発に協力した。

B.4.2. DA の開発協力（小島、足利）

OECD における皮膚感作性試験の DA の開発に協力した。

B.5. 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（チョウ）

6 週齢 SD ラット（雌雄各群 5 匹）に被験物質を経口投与し、28 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、放血による安楽死を行った。甲状腺、下垂体、卵巣、子宮、精巣及び副腎を用いて T3、T4、TSH、ACTH、calcitonin、β-actin、estrogen、progesterone、黄体形成ホルモン（LH）、卵胞刺激ホルモン（FSH）、prolactin、testosterone などの免疫染色による解析及び血中濃度測定を実施した。動物実験は対照群、1000 ppm aminotriazole （AMT）群、20 ppm vitamin D3 (VD3) 群、50 ppm propylthiouracil (PTU) 群、500 ppm phenobarbital (PB) 群、6000 ppm aminoglutethimide (AGT)群、10 ppm estradiol (E2)群の 7 群を設置した。AMT、PTU は飲水、VD3、PB、AGT、E2 は混餌投与した。投与量は過去に報告されている短・中期試験の最大耐量に基づき設定した。

B.6. 遺伝毒性の AOP 開発（杉山）

非遺伝毒性発がんの AOP への組み込みを想定した新規エピジェネティック変異原試験「FLO アッセイ」の妥当性評価に関する研究を行った。同試験系のプロトタイプは、既に開発している。今年度は遺伝毒性の判定は困難であるものの、国際がん研究機関より発がん性がクラス 2B に分類されているかび毒オクラトキシン A を被験物質に、同化学物質のエピジェネティック変異原性を検討した。

B.7. 腎障害の分子メカニズムに関する研究（松下）

10 週齢の雌雄 F344 ラット片側腎（左腎臓）摘出術を施し、残存腎における代償性肥大の分子機構を解明した。この代償性肥大は腎障害の発現機序に関わらず共通して生じる現象であることから、その発現機序を詳細に理解することにより、新しい腎障害評価分子を抽出することが期待できる。具体的には、雌雄ラットに片側腎摘出術を施して残存腎組織を採材し、細胞増殖活性を免疫組織学的及び遺伝子発現解析により検索し、網羅的遺伝子発現解析として、mRNA 及び microRNA(miRNA)の発現を解析した。

これにより、腎毒性の AOP 作成に資する情報を得る。

B.8. 光安全性試験の追加検討（尾上）

光毒性化合物である acridine (ACD)、furosemide (FSM)、hexachlorophene (HCP)、8-methoxypsoralen (MOP)、norfloxacin (NFX) 及び promethazine (PMZ) の UV 吸収測定及び ROS アッセイを実施した。Franz 型拡散セルを用いてラット摘出皮膚における被験物質 (各 1 mg/mL) の *in vitro* 皮膚透過性を評価し、定常状態における被験物質の皮膚内濃度を算出した。比較試験として、ラットを用いた *in vivo* 光毒性試験を実施し、被験物質 (10 mg/site) をラット腹部皮膚に塗布後の皮膚光毒性を評価した。

B.9. 毒性等情報収集（山田）

公募型研究で実施する化学物質の毒性等の情報をとりまとめ、整理された毒性等の情報に基づき、文献や報告書の検索の効率化や AOP 開発に資するため、以下の公共データベース及び厚労科研化学リスク

研究事業総合報告書情報を調査した。

1) NITE 化学物質総合情報提供システム (CHRIP;https://www.nite.go.jp/chem/chrip/chrip_search/systemTop)
2) PubChem(National Center for Biotechnology Information) (https://pubchem.ncbi.nlm.nih.gov/)
3) 厚労科研化学物質リスク研究事業 多色発光細胞を用いた high-throughput 免疫毒性評価試験法の開発 平成 24 年度～26 年度総合研究報告書（研究代表者 相場節也）

（倫理面への配慮）

本研究は動物実験の3Rsに配慮して、動物実験委員会の承認のもとに基本指針を遵守して実施し、動物使用数や動物に与える苦痛は最小限に留めた。ボランティア及びヒト組織は使用しなかった。これらのことから、倫理的問題は無いと考える。

C. 研究結果

C.1. AOP、TG、DA の開発、AOP 国内マニュアルの作成（小島）

AOP マニュアルを作成するため、EAGMST の資料を再調査し、以下の 2 報を用いることにした。

1) USERS' HANDBOOK SUPPLEMENT TO THE GUIDANCE DOCUMENT FOR DEVELOPING AND ASSESSING AOP
2) Revised Guidance Document on Developing and Assessing Adverse Outcome Pathways

C.2. TG 開発

C.2.1. TG の採択に向けた動向（小島、尾上）

1)皮膚感作性試験

皮膚感作性試験代替法 ADRA の TG 開発を、開発者の富士フィルムや国内外の専門家の協力を受け進めた。2 度に渡り、WNT から TG への意見を受け、修正して対応した。この結果、4 月の WNT 会議にて、採択が内定した。

2)光安全性試験

ROS アッセイの TG 開発を国内外の専門家と密な連携をとり進めた。WNT から TG への意見を受け、修正して対応した。この結果、4 月の WNT 会議にて、採択が内定した。

3)腐食性試験

LabCyte EPI-MODEL24 を用いる腐食性試験代替法の TG 開発を、開発者の株式会社 J-TEC と密な連携をとりつつ進めた。WNT から TG への意見を受け、修正して対応した。この結果、4 月の WNT 会議にて、採択が内定した。

C.2.2. 免疫毒性 TG の提案（小島、相場）

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 会議を 2019 年 2 月 27 日から 28 日まで、東京にて開催した。外部評価委員として Henk van Loveren (Maastricht University, Netherland), Haley LaNef Ford (Seattle Genetics, Inc., USA), Barbara Kaplan (Mississippi State University, USA), Sang-Hyun Kim (Kyungpook National University, Korea), Fujio Kayama (Jichi Medical University, Japan), Takao Ashikaga (National Institute of Health Sciences, Japan)を招請した。また, Xingchao Geng (National Center for Safety Evaluation of Drugs (NCSED), China)は電話会議にて参加した。

Peer review会議において，バリデーション報告書に対し、別紙１に示す提案が届き、次年度に対応予定である。

また、今後、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、本件に関する昨今の状況をまとめた DRP の SPSF を OECD に提案し（添付資料４）、2019 年 4 月の OECD　WNT 会議で作業計画として採択された。

C.2.3. 発がん性 TG の提案（小川）

ラット肝中期発がん性試験の TG 申請を行うために、関連論文等の資料を整理し、SPSF を OECD に提出した。この SPSF に対し、OECD 加盟各国からコメントがあり、対応して SPSF を改訂した。この結果、4 月の WNT 会議にて、作業計画への承認は見送られた。

C.2.4. Bhas42 細胞形質転換試験法の TG 開発（大森）

OECD NGTxC・IATA の専門家委員会にて、Bhas42CTA(プロモーション試験)の試験法テンプレート（Bhas 42 Cell transformation assay template （promotion test）を作成し、NGTxC assay template として提出した。6 月に OECD で開催された 3rd Meeting of the expert group for the development of an IATA for Non Genotoxic Carcinogen（OECD, 2018 年 6 月） では、”Bhas 42 cell transformation assay (Bhas 42 CTA) ”のプレゼンテーション実施し、Bhas42CTA 研究の背景と機序解析の進捗状況を報告した。また、形質転換試験と代謝協同阻害試験テンプレート作成を分担した。

C.3. AOPの開発

C.3.1. 免疫毒性のAOP開発（小島、足利、相場、久田）

1) AOP154 案 “Inhibition of calcineurin inhibition leading to impaired T cell-dependent antibody response”に関しては、EAGMST の内部 peer reviewer のコメントに対応して、主に key event relationship (KER)における定量的な理解（quantitative understanding）を中心に修正・追記した。6 月 28 日の EAGMST 会議において更新した AOP154 が内部 peer review を通過し、外部 peer review に進むこととなった。現在、外部 peer review に向けた内部 peer reviewer への指摘に対応している。

2) IL-2 を MIE とする AOP 案を AOP　wiki に入力した。来年度に内部 peer review に進むことを期待している。

3) 2018 年 2 月から、Janus kinase (JAK)-Signal Transducers and Activator of Transcription (STAT)、Aryl Hydrocarbon Receptor (AhR)、Estrogen receptor (ER)に対象を絞って文献調査を実施し、AOP の概要を検討した。その結果、MIE 及び AOをそれぞれ、１）JAK3 阻害及び TDAR 抑制、２）Toll-like receptor (TLR)7 活性化及び乾癬様皮膚炎発生、３）ER 活性化及びアレルギー性皮膚疾患の増悪、とする AOP の開発を決定し、それぞれ SPSF を作成して、11 月に OECD に提出した。これらの SPSF は 12 月 18 日の EAGMST 電話会議において協議され、ER 受容体活性化に関しては内容の再考が求められた。

C.3.2. 発がん性 AOP の開発（西川、小川、久田）

1) 細胞毒性を初動因子とした鼻腔発がんの AOP 開発を OECD に申請済であり、細胞傷害と腫瘍発生との関連に係るデータ解析を進めたところ、他の細胞毒性を初動因子とした AOP の成立が少ないことを鑑み、まずは、これまでに取りまとめたデータについて論文化を先行させる準備をしている。

2)ラットにおける非遺伝毒性発がん性 AOP の SPSF13 件(表 1)を作成し、研究代表者（小島）を介して OECD に提案したところ、12 月の EAGMST 会議において議論された。

表 1　本年度提案した発がん性 AOP

No	MIE　（機序）
1	トリプシン抑制
2	Vitamin D3 受容体活性化　（Ca 恒常性変化）
3	難吸収性炭水化物　（Ca 恒常性変化）
4	Sodium glucose cotransporter 1 阻害（Ca 恒常性変化）
5	Vesicular monoamine transporter（VMTA）阻害（レセルピン）
6	ドパミン D2 受容体阻害
7	プロトンポンプ阻害（高ガストリン）
8	H2 受容体阻害　（高ガストリン）
9	GLP-1 受容体活性化
10	α-glucosidase 阻害　（Ca 恒常性変化）
11	ドパミン D2 受容体阻害
12	難吸収性炭水化物　（Ca 恒常性変化）
13	ドパミン D2 受容体刺激

C.3.3. 光安全性の AOP 開発（尾上）

AOP 案を AOP　wiki に入力した。来年度に内部 peer review に進むことを期待している。

C.4. IATA 及び DA の開発

C.4.1. IATA の開発協力（西川、小川、大森）

6 月に OECD で開催された 3rd Meeting of the expert group for the development of an IATA for Non Genotoxic Carcinogen（OECD, 2018 年 6 月）にて、IATA の開発に協力した。

C.4.2. DA の開発協力（小島、足利）

OECD 専門家会議（電話会議や対面会議）で皮膚感作性 DA に関する TG の開発に寄与した。

現在、ヒト及び動物実験結果の再評価、適用限界の明確化、不確定要素の解決法について、それぞれサブワーキンググループが討議しており、その提案を待って DA の最終的な議論が来年度になされる予定である。

C.5. 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（チョウ）

既存のラット甲状腺及び下垂体のサンプルを用いて、ACTH（下垂体、抗原賦活化：クエン酸緩衝液 pH6.0（関東化学）、Autoclaving 121℃ 15min、希釈倍率：x16,000）、prolactin（下垂体、抗原賦活化：Antigen retrieval solution pH9.0 （Dako）、Autoclaving 121℃ 15min、希釈倍率：x8,000）、thyroxin（甲状腺、抗原賦活化：クエン酸緩衝液 pH6.0 （関東化学）、Autoclaving 121℃ 15min、希釈倍率：x2,000）

及び calcitonin（甲状腺、抗原賦活化：クエン酸緩衝液 pH6.0（関東化学)、Autoclaving 121℃ 15min、希釈倍率：x200）抗体の免疫染色条件を確立した。

今後は染色可能な抗体を増やすとともに、映像解析ソフトを用いた輝度の測定及びハウスキーピング蛋白による標準化方法を確立し、陽性細胞の輝度データが実際の血液中ホルモン濃度との相関を検討する。

C.6. 遺伝毒性の AOP 開発（杉山）

解析の結果、オクラトキシン A はエピジェネティック制御下にある酵母凝集遺伝子 *FLO1* の転写レベルを抑制することが明らかとなった。また、そのメカニズムとして DNA メチル化阻害が示唆される結果も得た。したがって、オクラトキシン A は 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が考えられる。

C.7. 腎障害の分子メカニズムに関する研究（松下）

雌雄ともに残存腎の組織学的解析では、処置後 2 及び 3 日において近位曲尿細管、近位直尿細管及び遠位尿細管の BrdU 陽性細胞率が対照群と比して有意な上昇あるいは上昇傾向を示した。また、雌雄ともに尿細管の細胞周期停止及び肥大に関わるとされている transforming growth factor (TGF)-β1 の mRNA 発現に処置による影響はみられなかった。mRNA 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雄では 320 個、雌では 233 個の遺伝子の発現が変動しており、それらの多くが細胞周期に関連する遺伝子の発現上昇であった。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（miR-1843a-5p、miR-1843a-3p、miR-194-3p、miR-222-3p、miR-31-5p、miR-340-5p、miR-450-5p、miR-653-5p、miR-9a-3p）発現が変動しており、全て発現低下を示した。

C.8. 光安全性試験の追加検討（尾上）

全被験物質は UVA/B 領域において高い UV 吸収特性ならびに擬似太陽光照射時に強い ROS 産生を認め、特に ACD は高い光反応性を示した。*In vitro* 皮膚内動態評価より、予測した皮膚中濃度は HCP 及び MOP がそれぞれ 101 及び 145 μg/mL と高く、FSM の皮膚中濃度は 3 μg /mL と最も低値を示した．光反応性及び *in vitro* 皮膚内動態の統合的解析より被験物質の光毒性リスクは HCP＞ACD＞MOP＞PMZ＞NFX＞FSM の順であると予測した。今回構築した *in vitro* 光安全性評価系により予測した光毒性リスクはラットにおける *in vivo* 光毒性の強さ (ACD＞HCP≒MOP＞FSM≒PMZ＞NFX) と良好に対応した。

C.9. 毒性等情報収集（山田）

収集した毒性情報は、Excel (Microsoft 社) 及び化学物質関連情報を収載するデータベースソフトウェアとして広く利用されている ChemFinder (Hulinks 社)、InstantJChem (ChemAxon 社)で閲覧・検索することを想定し、データ項目として、以下を仮設定した。

1) 化学物質情報（CAS No., 物質名称（日本語名、英語名）、SMILES（OECD

QSAR Toolbox, ChemSpider, PubChem 形式）、InChI Key 等）

2) *In vitro* バイオアッセイ情報（免疫毒性評価試験 MITA の情報とその元文献情報）
3) *In vivo* バイオアッセイ情報（免疫毒性；免疫抑制、免疫亢進、感作性とアレルギー反応、自己免疫と自己免疫疾患等情報とその元文献情報）
4) 国内外有害性評価書またはリスク評価書情報
5) 国内外法規制情報（化審法、化管法、安衛法、毒劇法、EU CLP 等）

C.10. 国際情報調査（稲若）

日本発の試験法を国際的なTGにする重要な情報として、OECDの試験法公定化の最新状況について調査した。また、国際機関等でのAOPやIATA開発の関連情報を収集した。

D. 考察

D.1. 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、今年度の OECD WNT 及び各国専門家との調整を通して、本年 4 月の WNT にて TG 案の採択が内定した。一年で 3 試験法を TG として採択できる機会は極めて稀であるが、最後まで気を引き締めて WNT との交渉に臨みたい。

腐食性試験代替法の TG431 に日本発の表皮モデルである LabCyte EPI-MODEL24 が加わることにより、毒物劇物の腐食性判定に代替法を用いることができる可能性が高くなる。TG431 が正式に改定され次第、行政的な受け入れに関する厚生労働省への働きかけを開始したい。

一方、新たに *in vitro* 免疫毒性 DRP の開発及びラット肝中期発がん性試験 TG のための SPSF を OECD に昨年 11 月に提出し、*in vitro* 免疫毒性 DRP は本年 4 月に WNT で採択された。引き続き、日本から OECD TG への働きかけを続けていきたい。

D.2 . 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 EAGMST における内部 peer review が終了し、外部 peer review が始まる。外部 peer review 終了まであと一息となった。来年度には必ず成立させたい。他にも光安全性、免疫抑制（IL-2 の発現）は AOP wiki の入力段階にあり、これらについては、来年度には内部 peer review に進むことを期待している。

新たに、免疫毒性で 3 件、非遺伝毒性発がん 13 件の SPSF を OECD に昨年末に提出しており、今後の種々の AOP を成立させることができると考えている。

D.3. IATA 及び DA の成立

非発がん性 IATA や感作性 DA 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 OECD に提案する予定であり、動物実験を用いない安全性評価の体系化を日本からも進めていく予定である。

D.4. TG 及び AOP の作成に寄与する実験

AOP 開発を支援する評価法の確立にお

いては、非遺伝毒性発がん検出のための甲状腺及び下垂体ホルモン等免疫組織化学染色条件の確立、エピジェニック変異原性のメカニズム解析、腎毒性における尿細管再生機構に関する解析を進めており、これらの成果を AOP 開発に生かせるような研究の進展を期待している。

まだ本研究班での検討は一年しか立っていないが、新たな知見が得られている。中間評価までには、成果を明らかにし、今後の TG や AOP への寄与のあり方を考えていきたい。

D.5. 毒性等情報収集

平成 30 年度終了予定の厚生労働科学研究費補助金化学物質リスク研究事業の総合研究報告書をもとにして、データベースのデータ項目の追加と再検討を行い、Excel のスプレッドシートのフォーマットでデータの入力を行う予定である。

E. 結論

昨年度からの OECD への継続した活動の中、本年度に TG や AOP が採択されたものはなかった。ただし、来年度に 3 試験法の TG、1 件の AOP を成立できる目途がたった。

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 TG の開発に協力していく予定である。

F. 健康危険情報

特になし

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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

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

平成30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 情報を活用する戦略がある。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 OECD の TG や AOP が採択されたものはなかった。ただし、来年度に 3 試験法の TG、1 件の AOP を成立できる目途がたった。

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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 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

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。

B.1.1. 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 Molecular initiating event（MIE）とし、T 細胞依存性抗体産生抑制（TDAR）を Adverse outcome（AO）とする AOP154 案 “Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response” を作成した。

B.1.2. 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、電話会議や専門家会議にて交渉した。

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 会議を企画した。

また、*in vitro* 免疫毒性試験に関する Detailed Review Paper(DRP)の SPSF を作成し、OECD に提案した。

B.1.3. DA の開発協力

足利分担研究者とともに、OECD における皮膚感作性試験の DA 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開発

AOP154 案 “Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response”に関しては、EAGMST の内部 peer reviewer のコメントに対応して、主に key event relationship (KER)における定量的な理解（quantitative understanding）を中心に修正・追記した。6 月 28 日の EAGMST 会議において更新した AOP154 が内部 peer review を通過し、外部 peer review に進むこととなった。現在、外部 peer review に向けた内部 peer reviewer への指摘に対応している。

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 の TG 開発を、開発者の富士フイルムや国内外の専門家の協力を受け進めた。2 度に渡り、WNT から TG への意見を受け、修正して対応した。結果として、2019 年 4 月の OECD WNT 会議で TG 案（添付資料 1）の採択が内定した。

2)光安全性試験

ROS アッセイの TG 開発を国内外の専門家と密な連携をとり進めた。WNT から TG への意見を受け、修正して対応した。

結果として、2019 年 4 月の OECD WNT 会議で TG 案（添付資料 2）の採択が内定した。

3)腐食性試験

LabCyte EPI-MODEL24 を用いる腐食性試験代替法の TG 開発を、開発者の株式会社 J-TEC と密な連携をとりつつ進めた。WNT から TG への意見を受け、修正して対応した。結果として、2019 年 4 月の OECD WNT 会議で TG 案（添付資料 3）の採択が内定した。

4)免疫毒性試験

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 会議を 2019 年 2 月 27 日から 28 日まで、東京にて開催した。外部評価委員として Henk van Loveren (Maastricht University, Netherland), Haley LaNef Ford (Seattle Genetics, Inc., USA), Barbara Kaplan (Mississippi State University, USA), Sang-Hyun Kim (Kyungpook National University, Korea), Fujio Kayama (Jichi Medical University, Japan), Takao Ashikaga (National Institute of Health Sciences, Japan)を招請した。また, Xingchao Geng (National Center for Safety Evaluation of Drugs (NCSED), China)は電話会議にて参加した。

Peer review会議において，バリデーション報告書に対し、別紙 1 に示す提案が届き、次年度に対応予定である。

また、今後、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、本件に関する昨今の状況をまとめた DRP の SPSF を OECD に提案し（添付資料４）、2019 年 4 月の OECD WNT 会議で作業計画として採択された。

C.1.3. DA の開発協力

OECD 専門家会議（電話会議や対面会議）で皮膚感作性 DA の開発に寄与した。

現在、ヒト及び動物実験結果の再評価、適用限界の明確化、不確定要素の解決法について、それぞれサブワーキンググループが討議しており、その提案を待って DA の

最終的な議論が来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 EAGMST における内部 peer review が終了し、外部 peer review が始まる。外部 peer review 終了まであと一息となった。来年度には必ず成立させたい。

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、OECD　WNT にて、2019 年 4 月に TG として採択されることが内定した。来年度の正式採択に向け、OECD と調整していきたい。

一方、新たに *in vitro* 免疫毒性 DRP の開発のための SPSF を OECD に昨年 11 月に提出し、2019 年 4 月に OECD 作業計画に加えられることになった。この DRP の開発を基に、TG の開発を目指すために OECD へ働きかけを続けていきたい。

D.3 . IATA 及び DA の成立

感作性 DA 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 OECD に提案する予定であり、動物実験を用いない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 OECD との継続した活動の中、本年度に TG や AOP が採択されたものはなかった。ただし、来年度に 3 試験法の TG、１件の AOP を成立させることができる目途がたった。

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。

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G. 知的所有権の取得状況

G.1 特許取得

特になし

G.2. 実用新案登録

特になし

G.3 その他

特になし

H. 添付資料

1. OECD Draft Updated Test Guideline 442C for in chemico skin sensitisation assays addressing the Adverse Outcome Pathway Key Event on covalent binding to proteins
2. OECD GUIDELINE FOR THE TESTING OF CHEMICALS: ROS (REACTIVE OXYGEN SPECIES) ASSAY FOR PHOTOTOXICITY
3. OECD DRAFT UPDATED TEST GUIDELINE 431 ON INVITRO SKIN CORROSION, RECONSTRUCTED HUMAN EPIDERMIS TEST METHODS

別紙 1　Action Items to peer reviewers for the validation report on the IL-2 Luc assay

Evaluation Criterion 1: A rationale for the test method should be available, including a description of the human health effect, a clear statement of scientific need, and regulatory application.

PRP Comment: Together with a new title, the rationale needs to be stated clearly to be T-cell targeting.

Evaluation Criterion 2: The toxicological mechanisms and the relationship between the test method endpoint(s) with the biological effect as well as the toxicity of interest should be addressed, describing limitations of the test method.

PRP Comment: Needs to focus on IL-2, including the limitations described in the meeting minutes. The introduction needs to focus solely on IL-2 and the IL-2 Luc Assay. Discussion about its part in MITA should be left until the discussion section.

Evaluation Criterion 3: A detailed test method protocol should be available

PRP Comment: The commercial availability of the #2H4 cell line needs to be described.

Evaluation Criterion 4: The within and between laboratory reproducibility of the test method should be demonstrated

PRP Comment: Acceptable

Evaluation Criterion 5: Demonstration of the test method's performance should be based on testing of representative, preferably coded reference chemicals

PRP Comment: We think only four or five negatives is not enough, so we suggest that some additional testing of negatives be performed.

Evaluation Criterion 6: Predictive capacity should be demonstrated using representative chemicals.

PRP Comment: Predictive capacity needs to be reassessed based on today's proposed definition of T-cell–targeting chemicals.

Evaluation Criterion 7: All data should adequately support the assessment of the validity of the test method for peer review.

PRP Comment: A clear definition of the 35% threshold and a clear explanation of Criteria 5 and how it was developed is needed. Should the table in Appendix 8 include the test judgment? Also, delete DTH, tumor, infection, and NK activity but specify T-cell proliferation in the table in Appendix 8.

Evaluation Criterion 8: All data from the validation study supporting the validity of a test method should be obtained in accordance with the principles of Good Laboratory Practice (GLP)

PRP Comment: The report needs to explain clearly and in detail what is meant by the phrase "in the spirit of GLP" and whether or not each laboratory performed their work in this spirit.

Evaluation Criterion 9: Applicability domain of the test method should be defined

PRP Comment: We recommend that the applicability domain be more clearly defined as noted in the PRP meeting minutes.

Evaluation Criterion 10: Proficiency chemicals should be set up in the proposed protocol

PRP Comment: None

Evaluation Criterion 11: Performance standards should be set up with the proposed protocol

PRP Comment: If performance standards are understood to be assay controls, then the use of three-fold stimulation of IL-2 Luc by PMA/IO and inhibition of stimulated IL-2 Luc by DEX and CYA are sufficient. We suggest that acceptance criteria for variability within test replicates be defined.

Evaluation Criterion 12: Advantages in terms of time, cost and animal welfare

PRP Comment: We suggest that the conclusion leave out mention of in vivo testing to confirm T-cell immunotoxicity and include discussion of the use of IL-2 Luc assay within MITA.

Evaluation Criterion 13: Completeness of all data and documents supporting the assessment of the validity of the test method.

PRP Comment: We suggest that data be redone to reassess predictive capacity based on today's proposed definition of T-cell–targeting chemicals. Also, a critical assessment of the 35% threshold in the context of the new definition of T-cell targeting is necessary.

Evaluation Criterion 14: Validation Study Management and Conduct

PRP Comment: None

Other considerations

PRP Comment: None

Conclusion

PRP Comment: We look forward to seeing a revised report based on our comments.

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.

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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	Farnesol	4602-84-0	Liquid	222.37	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 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.

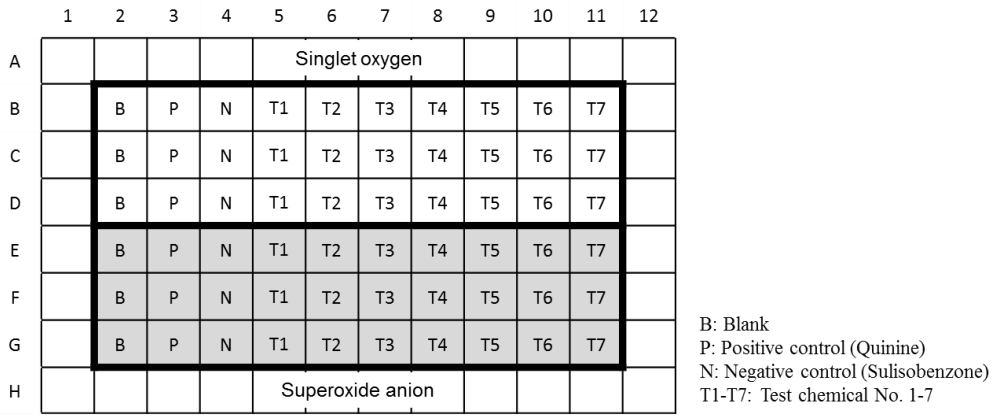
Positive and negative controls

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

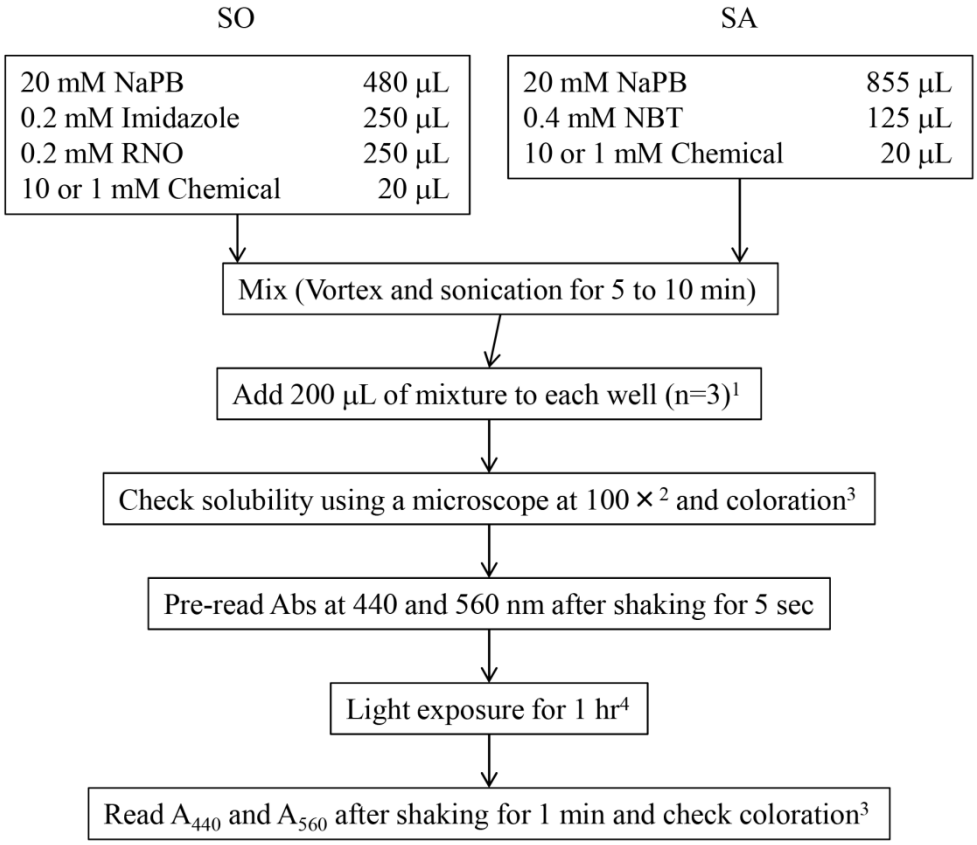
23. A typical 96-well plate configuration is as follows, but other configurations are also acceptable:

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.



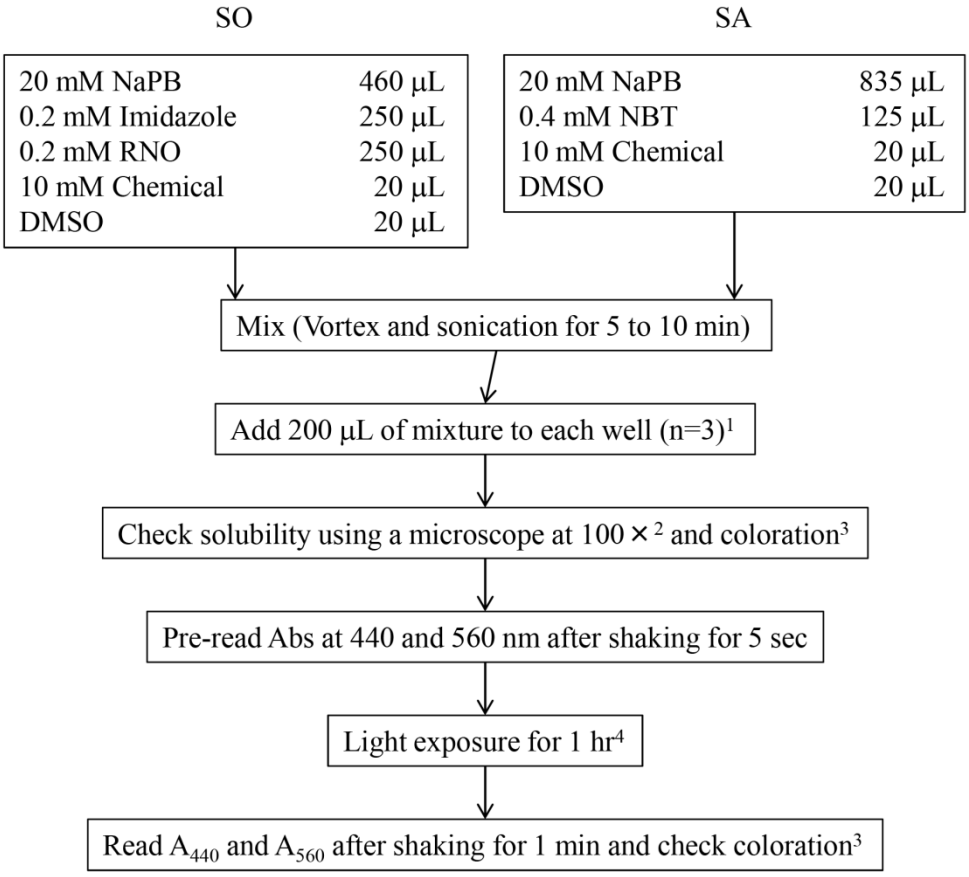
[1] Avoid using peripheral wells. More than one test chemical can be tested on a plate.

[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.
-

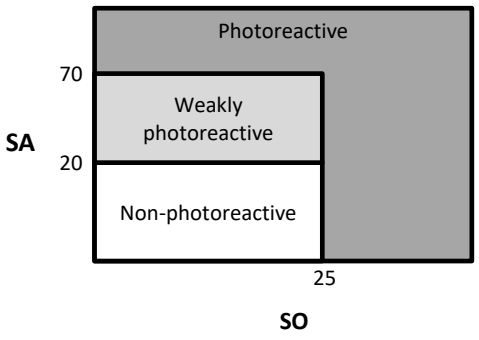
  Positive control (quinine hydrochloride) value at 200 µM (mean of 3 wells)
  SO: 319 to 583
  SA: 193 to 385
  Negative control (sulisobenzone) value at 200 µM (mean of 3 wells)
  SO: -9 to 11
  SA: -20 to 2
- Laboratories should demonstrate technical proficiency, as described in Annex C, prior to routine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

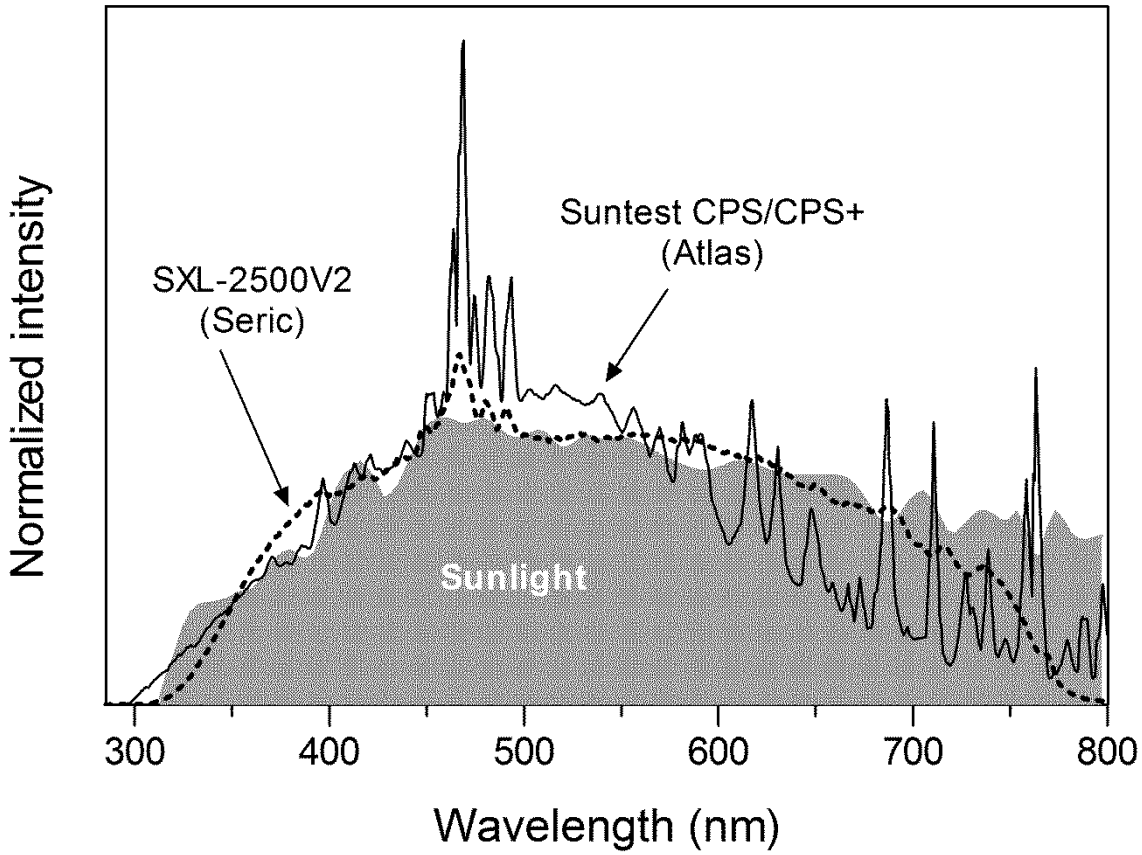
ROS: Reactive Oxygen Species, including superoxide anion (SA) and singlet oxygen (SO).

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).

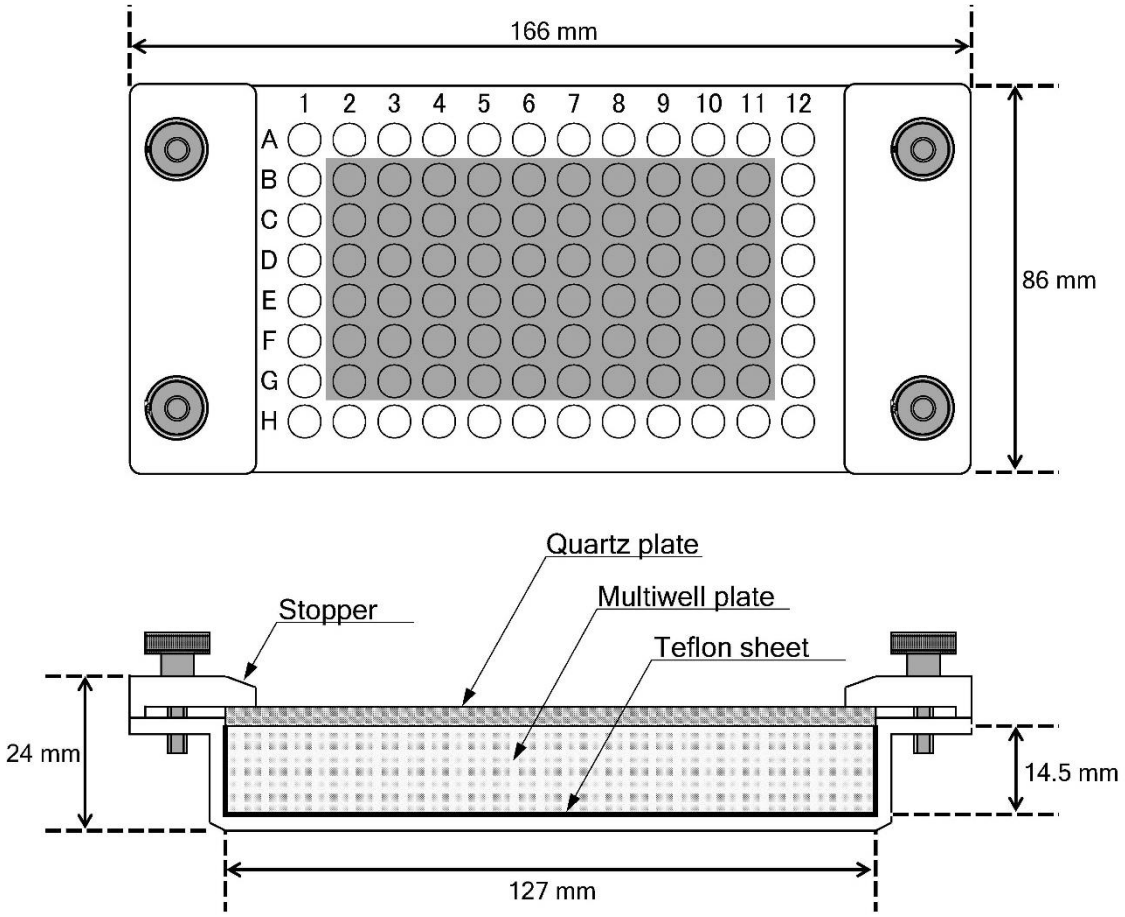
Table A C.1. Table of proficiency chemicals.

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.

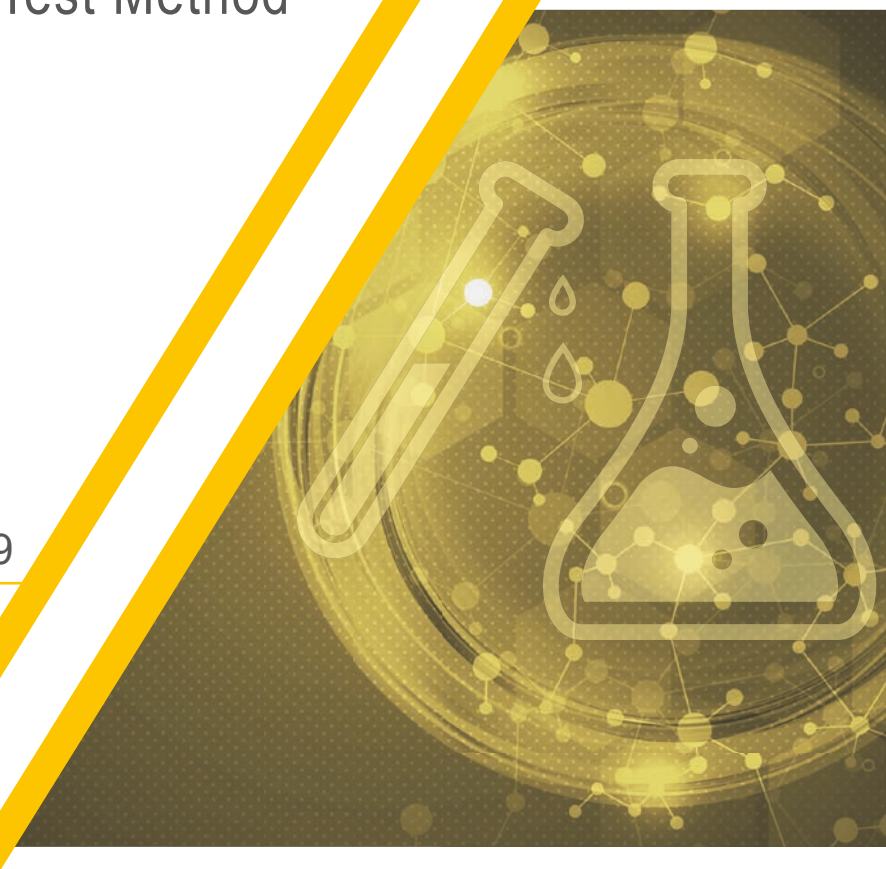


Recommended thickness of quartz plate: ca. 3 mm.

Section 4
Health effects

Test Guideline No. 431

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



18 June 2019

OECD Guidelines for the Testing of Chemicals

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 3 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.

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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

平成30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の TG 申請を行うため、文献資料を整理し作成した SPSF (Standard Project Submission Form) を OECD に提案した。また、2013 年から 2017 年に厚労省の依頼によって 3 つの施設で実施された合計 26（それぞれ 10，8，8 試験）の試験結果について、溶媒を投与した陰性対照群及びフェノバルビタールを投与した陽性対照群のデータを比較検討し、本法の再現性などを検討したところ、何れの施設においてもデータは安定しており、陽性対照群は有意差をもって検出されていることが確認された。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 を OECD に提案した。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，今年度はその起始分子イベントを検討した。その結果，酸化的ストレスが候補として浮上したため，それを起始イベントとして AOP 作成を継続する。同時に、これまで検討してきた鼻腔発がんの病理発生に係る情報を整理し、論文投稿を目指す。

ラット肝中期発がん性試験及びラットにおける非遺伝毒性発がん性AOPに関するSPSFに対して、OECD加盟各国からコメントが得られた。これらに対応したSPSFの改訂版を提出し、今後、TG化あるいはガイダンスドキュメント作成及びAOPの作成を進める予定である。また、非遺伝毒性発がん性のIATA作成について，2018年6月にOECDにて開催

された非遺伝毒性発がん性IATA作成専門家会議に出席し、討議に参画した。非遺伝毒性発がん性に係る試験・検査のパラメータを優先順位別に4つのカテゴリーに分けること、候補となる試験・検査法を13のブロックに分け、分担してレヴューすることに合意した。

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の TG 申請を行うため、文献資料を整理し作成した SPSF (Standard Project Submission Form) を OECD に提案する。また、2013 年から 2017 年に厚労省の依頼によって 3 つの施設で実施された合計 26（それぞれ 10, 8, 8 試験）の試験結果について、溶媒を投与した陰性対照群及びフェノバルビタールを投与した陽性対照群のデータを比較検討し、本法の再現性などを検討する。

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（久田茂ほか）の協力を得て、ラットにおける非遺伝毒性発がん性 AOP にかんする SPSF を OECD に提案する。

（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する文献を PubMed および NTP のデータベースを用いて網羅的に検索し、鼻腔腫瘍の組織型と非巣陽性病変、投与経路並びに遺伝毒性との関連性について、さらに解析を進めた。OECD に AOP 案を提出した際に発がんの起始分子イベントを細胞障害としてよいとの回答を得ているが、さらに適切な分子イベントがないか検索する。

B.3. 非遺伝毒性発がん性の IATA 作成への協力（西川・小川）

2018年6月24日～29日にOECDにて開催された非遺伝毒性発がん性IATA作成専門家会議に出席し、討議に参画した。

（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 TG 申請を行うために、関連論文等の資料を整理して SPSF を作成し、厚労省を介して OECD に提案した。SPSF 提案に対する 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。

2013 年から 2017 年に 3 つの施設で実施された 26 の試験結果については、何れの施設においてもデータは安定しており、陽性対照群は有意差をもって検出されていることが確認された。

C.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 の SPSF を 13 件作成し、研究代表者（小島）を介して OECD に提案した。12 月の EAGMST 会議において議論された。最も大きなコメントは、その AOP の全てがげっ歯類に特異的な経路であって、ヒトに外挿できないのであれば、AOP には馴染まないという意見であった。いずれもヒトでの発がん性は低いと考えられるが、外挿できないとまでは言えない点を強調して、SPSF の改訂をおこない OECD に提出した。

（2） 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ことから、今後はその方向で作成を進める。同時に、これまでの病理学的検討は鼻腔発がんの病理発生解明に大きく寄与するはずであり、別途論文化を目指して準備をしている。

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 策定に協力した。6 月の会議において、非遺伝毒性発がん性に係る試験・検査のパラメータを優先順位に関して 4 つのカテゴリーに分けることにした。また、候補となる試験・検査法を 13 のブロックに分けて、分担してレヴューすることになった（添付資料参照）。

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 TG 申請を目差して SPSF を申請した。提案をサポー

トしないとのコメントに対しては、文献を用いて説明を追加して改訂版の SPSF を作成した。4 月の WNT 会合においても、本試験法の意義について説明をおこない TG 化あるいはガイダンスドキュメント作成を目差す。

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 に沿って、順次、AOP 案を作成する。ラット特異的か、あるいはヒトへの外挿性も考慮すべきか慎重に取りまとめる必要がある。

（2） 細胞毒性を初動因子とした鼻腔発がんの AOP 開発を OECD に申請済であり、細胞傷害と腫瘍発生との関連に係るデータ解析を進めたところ、他の細胞毒性を初動因子とした AOP の成立が少ないことを鑑み、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）

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 化を目指して SPSF を作成し OECD に提出した。得られたコメントに対して回答集及び改訂版を作成し再提出した。

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のSPSFを13件作成し、OECDに提案した。得られたコメントに対して改訂版を作成し再提出した。今後ヒトへの外挿性を考慮して順次AOPを作成する。

（2）細胞毒性を初動因子とした鼻腔発がんのAOP開発に関して検討し、今後、論文化を進める。

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作成を継続した。

F. 研究発表

F.1. 論文発表

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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

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

平成30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

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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増加と関連しているものが多い。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において、triiodothyronine（T3）、T4 や TSH などの測定を決定したことから、各種ホルモンレベル測定の重要性は認識されているが、より感度の高い測定方法、あるいは、血清以外を用いたホルモンの解析方法が必要と考えられる。30 年度においては、ラットを用いてホルモン濃度が亢進している陽性臓器の病理組織標本のサンプルセットを作製するとともに、既存のサンプルを用いて染色条件を検討した。その結果、雌雄の aminotriazole（AMT）群及び propylthiouracil（PTU）群で T3 及び T4 の有意な低値、aminoglutethimide（AGT）群で副腎皮質刺激ホルモン（Adrenocorticotropic hormone、ACTH）の有意な高値が認められ、作製したサンプルセットが今後の検討に有用であることが示された。AMT 群、PTU 群及び AGT 群の全例で甲状腺の肥大/過形成並びにコロイドの枯渇が認められた。AMT 群及び PTU 群の全例で副腎皮質の萎縮が、AGT 群の全例で副腎皮質のびまん性空胞化が認められた。正常ラットの甲状腺における T4、calcitonin 並びに下垂体における ACTH、PRT の免疫染色条件を確立した。今後は染色可能な抗体を増やすと共に映像解析ソフトを用いた輝度の測定及びハウスキーピング・タンパクによる標準化の方法を確立し、陽性細胞の輝度データが、実際の血液中ホルモン濃度と相関するか検討を進める。

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増加と関連しているものが多い。有害性発現経路（AOP : Adverse Outcome Pathway）には、一つの標的分子への作用（Molecular Initiating Event, MIE）及び重要な事象（Key Event、KE）が測定可能であることが必須とされていることから、血中 PRL、TSH 及び T4 は非遺伝毒性の発がん機序における MIE 又は KE になり得ると考えられる。更に近年、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において、triiodothyronine（T3）、T4 や TSH などの測定を決定したことから、毒性の意義やヒトへの外挿性を考慮する上でも、

重要な指標ではあるが、通常の方法（ECLIA)によるホルモンの測定にホルモンの測定には 0.4～0.5 ml の血清が必要であり、ラットでは可能であるが、マウスでは困難である。より感度の高い測定方法、あるいは、血清以外を用いたホルモン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定量的検出法の確立を試みる。通常の動物実験で得られる標本から検討可能な免疫染色を用いて検討を進める。30 年度においては、ラットを用いてホルモン濃度が亢進している臓器の病理組織標本のサンプルセットを作製するとともに、既存のサンプルを用いて染色条件を検討した。

B. 研究方法

既存のラット甲状腺及び下垂体のホルマリン固定、パラフィン包埋サンプルを用いて、ACTH（Peninsula 社 #T-4002、rabbit polyclonal）、prolactin（Abcam 社 #ab183967、rabbit monoclonal、clone EPR19386）、thyroxin（Novusbio 社 #NB110-7978、mouse monoclonal、clone XM212）、calcitonin（GeneTex 社 #GTX28553、rabbit polyclonal）、TSH（BIO-RAD 社 #8924-1709、rabbit polyclonal）、triiodothyronine（MyBioSource 社 #MBS2025743、mouse monoclonal）及びβ-actin（Sigma-Aldrich 社 #A2228-100UL mouse monoclonal、clone AC-74）抗体の免疫染色条件（希釈倍率、抗原賦活化有無及び条件）を検討した。

動物実験は基礎食（Basal diet）群、1000 ppm aminotriazole（AMT）群、20 ppm vitamin D_3（VD3）群、50 ppm propylthiouracil（PTU）群、500 ppm phenobarbital（PB）群、6000/1500 ppm aminoglutethimide（AGT）群、10 ppm estradiol（E2）群の 7 群を設置した。AMT、PTU は飲水、VD3、PB、AGT、E2 は混餌投与した（Fig. 1）。投与量は過去に報告されている短・中期試験の最大耐量に基づき設定した。6 週齢 SD ラット（雌雄各群 5 匹）に被験物質を経口投与し、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、HE 標本を作製し、病理組織学的検討を行った。

血液学的検査として、赤血球数（RBC）、ヘモグロビン量（HGB）、ヘマトクリット値（HCT）、平均赤血球容積（MCV）、平均赤血球血色素量（MCH）、平均赤血球血色素濃度（MCHC）、網状赤血球比率（RET）、血小板数（PLT）、白血球数（WBC）を、白血球分類においては好中球（NEUT）、リンパ球（LYMPH）、単球（MONO）、好酸球（EO）、好塩基球（BASO）の各比率を多項目自動血球計数装置（ProCyte DX、IDEXX laboratories）により測定および算出した。

血清生化学的検査として、総タンパク（TP）、アルブミン/グロブリン比（A/G）、アルブミン（ALB）、総ビリルビン（T-BIL）、グルコース（GLU）、トリグリセリド（TG）、総コレステロール（T-CHO）、尿素窒素（BUN）、クレアチニン（CRE）、ナトリウム（Na）、クロール（Cl）、カリウム（K）、カルシウム（Ca）、無機リン（IP）、アスパラギン酸トランスアミナーゼ（AST）、アラニントランスアミナーゼ（ALT）、アルカリフォスファターゼ（ALP）、γ-グルタミルトランスペプチダーゼ（γ-GT）の各項目を測定した。また、T3、T4、TSH、ACTH、卵胞刺激ホルモン（Follicle stimulating hormone、FSH）、黄体化ホルモン（Luteinizing hormone, LH）、PRL、estradiol、progesterone、testosterone などの血清中濃度測定を実施した。

統計解析

データは Microsoft Excel により集計し

Basal diet 群を基準とした Dunnett の検定を行い、$p<0.05$ 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. 免疫染色条件確立（Fig. 2）

既存のラット甲状腺及び下垂体のサンプルを用いて、ACTH（下垂体、抗原賦活化：クエン酸緩衝液 pH6.0（関東化学）、Autoclaving 121℃ 15min、希釈倍率：x16,000）、prolactin（下垂体、抗原賦活化：Antigen retrieval solution pH9.0 （Dako）、Autoclaving 121℃ 15min、希釈倍率：x8,000）、T4（甲状腺、抗原賦活化：クエン酸緩衝液 pH6.0（関東化学）、Autoclaving 121℃ 15min、希釈倍率：x2,000）及び calcitonin（甲状腺、抗原賦活化：クエン酸緩衝液 pH6.0（関東化学）、Autoclaving 121℃ 15min、希釈倍率：x200）抗体の免疫染色条件を確立した。ACTH は下垂体前葉の好塩基性細胞と中間葉細胞が、PRL は下垂体前葉の好酸性細胞が、T4 は甲状腺濾胞上皮と一部の腺腔内のコロイドが、calcitonin は甲状腺 C 細胞が陽性を示した。

C.2. 一般状態および死亡動物

29-30 日間の試験期間を通じ、死亡動物は認められなかったが、6000 ppm AGT 群で著しい体重増加抑制が認められ、投与量を 1500 ppm に変更した。

C.3. 体重および摂餌量（Fig. 3）

雌雄 AGT 雄及び雄 E2 群において、投与 1 週目以降、雌雄 VD3 群及び雄 PTU 群において、投与 2 週目以降、雌雄の AMT 群及び雌の PTU 群において、投与 3 週目以降、Basal diet 群に比較して有意な体重増加抑制が認められた。投与 1 週目の雄 PB 群及び 2 週目の雌 E2 群において Basal diet 群に比較して各々有意な体重増加及び減少認められた。摂餌量については、
雌雄 AGT 群で、投与 2 週間、低い傾向が認められ、飲水量については、雌雄 VD3 群で、投与 2 週間、高い傾向が認められた。

C.4. 血液学的、血清生化学的及びホルモン濃度検査（Table 1、2、3）

血液学的検査では、雄の AMT、VD3 及び PTU 群で MCHC の増加及び RET 及び PLT の低下が認められた。AMT 群で RBC、HGB 及び HCT、VD3 群で RBC、PTU 群で HGB、BASO 及び AGT 群で RET の増加が認められた。PTU 群の WBC、AGT 群の RBC、HGB 及び HCT の低下が認められた。雌の AMT 及び PTU 群で MCHC の増加及び RET の低下が認められた。AMT 及び VD3 群で MCV、PTU 群で PLT、PB 群で BASO、AGT 群で MCHC の低下が認められた。AGT 群で MCV 及び RET の増加が認められた。

血清生化学的検査において、雄 AMT 及び PTU 群の ALB、T-CHO、BUN、CRE の増加及び TG 及び IP の低下が認められた。AMT 群で T-BIL、ALT、VD3 群で Ca、PTU 群で TP、PB 群で IP、AGT 群で T-CHO、γ-GT の増加が認められた。VD3 群で TG 及び K、PTU 群で K、AGT 群で TG、Cl、K、E2 群で AST の低下が認められた。雌の AMT、PTU 群で BUN、CRE の増加及び IP の低下が認められた。AMT、VD3 及び AGT 群の TG の有意な低下が認められた。VD3 群の TP、ALB、AGT 群の Cl、K、AST、E2 群の Na の低下及び VD3 群の Ca 及び AGT 群の T-CHO、γ-GT の増加が認められた。

血清中のホルモン濃度検査において、雌雄 AMT 及び PTU 群の T3、T4 の有意な低下及び AGT 群の ACTH の有意な増加が認められた。雄 E2 群で T3、T4 の増加及び AGT 群で T3 の低下が認められた。何れの群の TSH も測定限界以下（0.005 μIU/mL）であった。

C.5. 臓器重量（Table 4）

雌雄の AMT 群及び PUT 群の甲状腺、PB 群の肝臓の絶対・相対重量の増加が認められた。雌雄の AMT 群及び PUT 群の副腎、肝臓、腎臓の絶対重量の減少、VD3 群の下垂体及び肝臓の絶対重量の減少が認められた。雌雄 AMT 群の肝臓の相対重量の減少及び VD3 群の副腎、腎臓の相対重量の増加が認められた。雄 AMT 群、VD3 群、PTU 群、AGT 群及び E2 群の精巣の相対重量の増加が認められた。雄 E2 群の副腎の絶対重量増加及び AGT 群の下垂体、腎臓、E2 群の肝臓、腎臓の絶対重量の減少が認められた。雄 AMT 群、PTU 群の下垂体、AGT 群の副腎及び肝臓、E2 群の副腎の相対重量の増加が認められた。雌 AGT 群の甲状腺の絶対・相対重量の増加が認められた。雌 AGT 群の肝臓絶対重量、腎臓の相対重量の増加が認められた。VD3 群の卵巣絶対重量の低下、PTU 群の副腎、肝臓の相対重量の低下が認められた。

C.6. 病理組織学的検索（Fig. 4、Table 5）

雌雄の AMT 群、PTU 群及び AGT 群の全例において、甲状腺濾胞上皮の肥大、過形成、コロイドの枯渇及び下垂体前葉細胞の肥大・空胞化が認められた。雌雄の AMT 群及び PTU 群の全例において、副腎皮質の萎縮が認められ、AGT 群の全例において、副腎皮質の空胞化が認められた。雌雄 PB 群の全例において、甲状腺濾胞上皮の肥大が認められた。雄の PB 群の 5 例中 4 例で下垂体前葉細胞の肥大及び空胞化が観察された。雌の PB 群の 5 例中 1 例で下垂体前葉細胞の肥大が認められた。雄 AMT 群の 5 例中 1 例で副腎皮質の過形成が認められた。雄 VD3 群の 5 例中 1 例で嚢胞及びラトケ嚢遺残が観察された。

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定量的検出法の確立を試みており、30 年度においては、ラットを用いてホルモン濃度が亢進している臓器の病理組織標本のサンプルセットを作製するとともに、既存のサンプルを用いて染色条件を検討した。

既存のラット甲状腺及び下垂体のサンプルを用いて、ACTH（下垂体前葉細胞）、PRL（下垂体前葉細胞）、T4（甲状腺濾胞上皮）、calcitonin（甲状腺 C 細胞）抗体の免疫染色条件を検討したところ、各々標的とする細胞に陽性反応が認められたことから、上記 4 つの抗体について免疫選諸条件の確立が出来た。実験動物に用いられる免疫染色用の抗体は多く知られているが、ホルモンの抗体は数が少ない（Furukawa S et. al., J Toxicol Pathol. 2016;30:79–107）。定量的検出法の確立を考慮すると入手が容易でホルマリン固定・パラフィン包埋標本に確実に染まる抗体が望ましい。

除草剤である AMT を投与した群において、雌雄 T3、T4 の低値、甲状腺重量の高値、甲状腺濾胞上皮の肥大、過形成、コロイドの枯渇、下垂体前葉細胞の空胞化及び副腎皮質の萎縮が認められた。AMT はラットにおいて抗甲状腺作用を示すことが知られており、甲状腺ホルモン、甲状腺濾胞上皮の病変は AMT 投与の影響と考えられた（Jukes TH et. al., Science 1960;132:296–7）。下垂体の病変は T3、T4 の低下に影響を受けた向甲状腺細胞に起因したと考えられるが、血清中の TSH の濃度が測定限度以下であったことで変化の確認が出来なかったことから、検出感度の優れた方法で再検討を行う必要があ

ると考えられた。TSH に加え、FSH、LH、PRL 及び testosterone の再検討も望ましい。甲状腺機能低下症は副腎皮質の萎縮及び副腎皮質ホルモン産生抑制を誘導することが知られている（Jukes TH et. al., J Embryol Stem Cell Res 2018;2:1–5）。AMT と共にチロシン残基とヨウ素結合を阻害することで甲状腺作用を示す、甲状腺機能亢進症治療薬 PTU を投与したラットにおいて、AMT 投与群と殆ど同じ甲状腺ホルモン濃度、臓器重量及び病理組織学的所見が認められた。抗てんかん薬である PB は肝ミクロソーム酵素を誘導し甲状腺障害作用を示す。PB 投与群では雌雄ラットの肝臓絶対・相対重量の増加及び甲状腺濾胞上皮に肥大、雄ラットの下垂体前葉細胞に空胞化及び肥大が認められた。雌の下垂体において、明らかな病変が認められなかったのは PB の薬力学における性差の可能性が考えられた（Hoffman A et. al., 大・空胞化及び副腎皮質の萎縮が認められた。AGT は下垂体前葉 ACTH 産生細胞の過形成を誘導することが知られている（Zak M et. al., Virchows Arch B Cell Pathol Incl Mol Pathol 1985;49:93‐106）。AGT は ACTH 産生細胞のみならず、性腺刺激ホルモン分泌細胞及び向甲状腺細胞を刺激し、過形成を誘導することから、甲状腺等の病変も誘発したと考えられる。

性ホルモンである E2 を投与したラットにおいて、雄 T3、T4 の 高値が認められた。下垂体の明らかな病理組織学的所見が認められなかった原因は、摂餌量の低下による被験物質のばく露量の減少傾向であると考えられた。

今後は染色可能な抗体を増やすと共に映像解析ソフトを用いた輝度の測定及びハウスキーピング・タンパクによる標準化の方法を確立し、陽性細胞の輝度データが、実際の血液中ホルモン濃度と相関するか検討を進める予定である。

E. 結論

本分担研究では、非遺伝毒性の発がん機Pharmaceutical Research 1989;6:976–81）。T3、T4 及び TSH を標的とするサンプルセットの作製は遂行できた。

高カルシウム血症を誘導することで甲状腺 C 細胞に過形成を誘導する VD3 を投与した群では雌雄の血清中 Ca 濃度の有意な増加が認められたものの、甲状腺 C 細胞の明らかな病理組織学的所見は認められなかった。1 ヶ月以上 VD3 を投与した実験の結果から、高カルシウム血症にもかかわらず、VD3 中毒が原因で甲状腺 C 細胞の肥大・過形成が認められないとの報告があった（Martin Lacave I et. al., J Endocrinol Invest 1998;21:102–8）。

副腎皮質抑制薬 AGT を投与した雌雄ラットにおいて、血清 T3 の低下又は低下傾向、血清 ACTH 濃度の増加、甲状腺重量の増加又は増加傾向、甲状腺濾胞上皮の肥大・過形成、コロイドの枯渇、下垂体前葉細胞の肥序に関連する、PRL、TSH、T4 を含む各種ホルモンレベルの簡便な定量的検出法の確立を試みる。30 年度においては、病理組織標本のサンプルセットを作製するとともに、既存のサンプルを用いて染色条件を検討した。ラット甲状腺及び下垂体のサンプルを用いて、ACTH（下垂体前葉細胞）、PRL（下垂体前葉細胞）、T4（甲状腺濾胞上皮）、calcitonin（甲状腺 C 細胞）抗体の免疫染色条件を確立した。又、明らかなホルモン濃度の変動及び病理組織学的病変を示すサンプルセットを作製することが出来たが、一部のホルモンは検出感度の優れた方法での再検討が望まれた。今後は染色可能な抗体を増やすと共に映像解析ソフトを用いた輝度の測定及びハウスキーピング・タンパクによる標準化の方法を確立し、陽性細胞の輝度データが、実際の血液中ホルモン濃度と相関するか検討を進める。

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

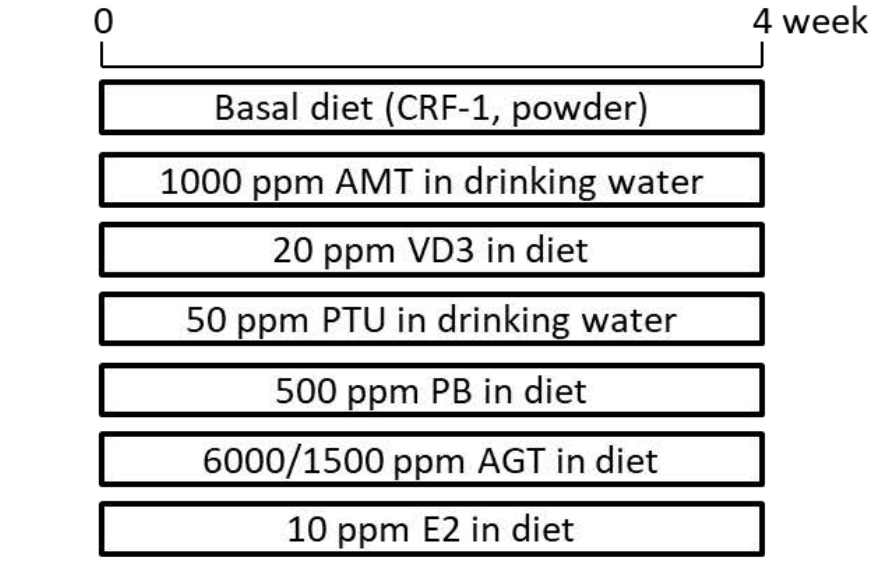

Figure 1. 実験デザイン

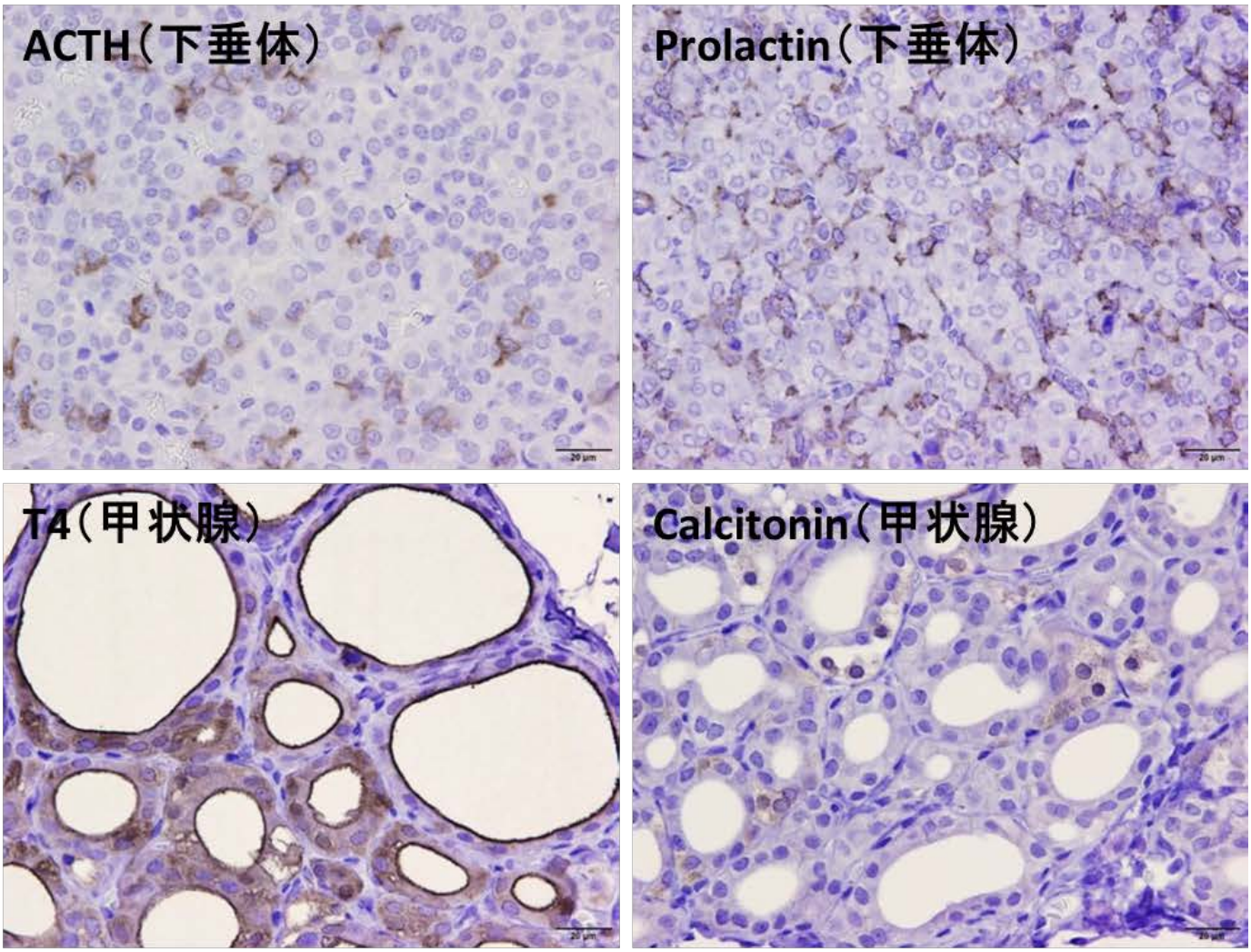

Figure 2. 既存サンプルを用いた免疫染色

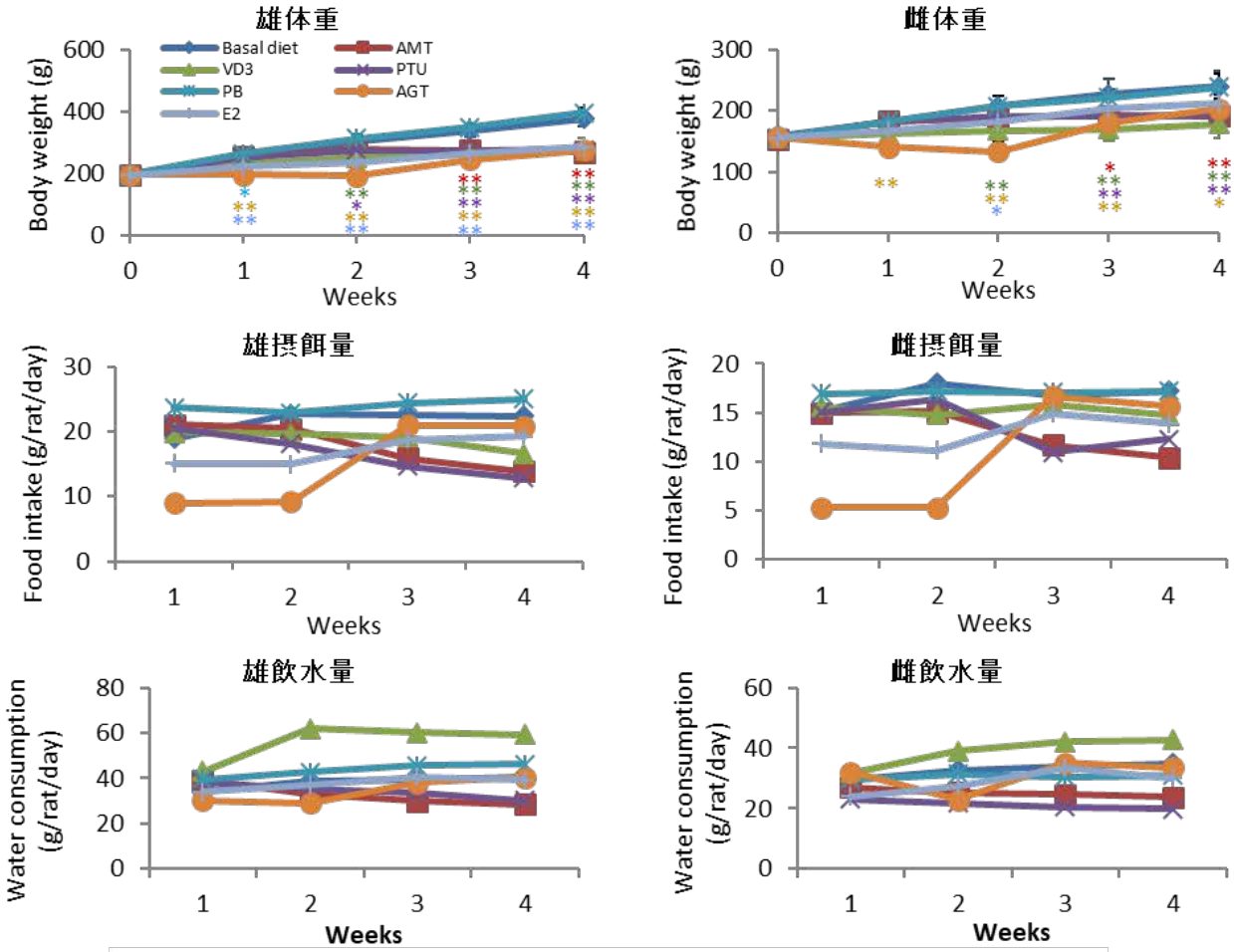

Figure 3. 体重、摂餌量、飲水量

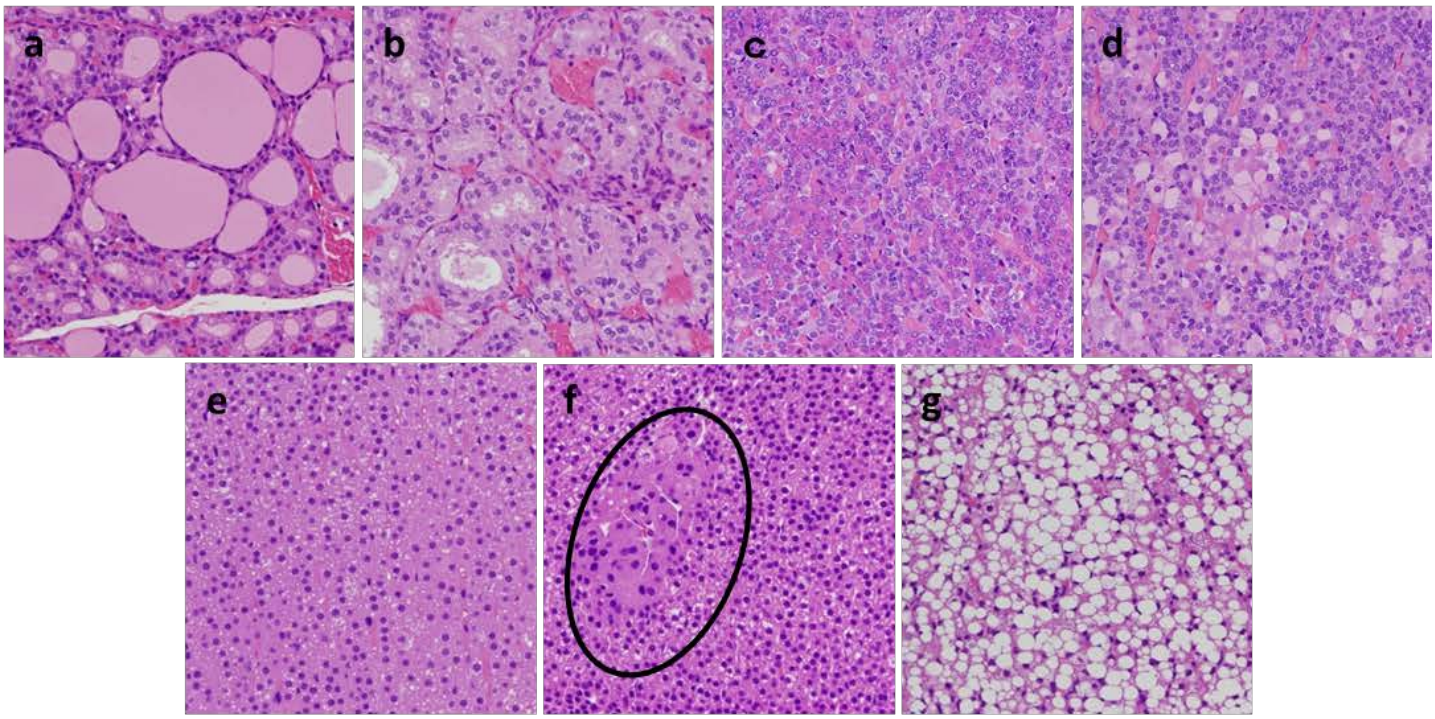

Figure 4. 病理組織像

a 正常甲状腺（対照群）、**b** 甲状腺の肥大、過形成及びコロイドの枯渇（AMT群）、
c 正常下垂体（対照群）、**b** 下垂体前葉の空胞化（AMT群）、
e 正常副腎皮質（対照群）、**f** 副腎皮質過形成（円内）及び萎縮（AMT群）、**g** 副腎皮質の空胞化（AGT群）

Table 1. Hematology data for SD rats

		Basal diet	AMT	VD3	PTU	PB	AGT	E2
Male								
No. of animals		5	5	5	5	5	5	5
RBC	$\times 10^6/\mu l$	7.4 ± 0.3	8.5 ± 0.5 **	8.2 ± 0.6 *	8.1 ± 0.6	7.5 ± 0.3	6.5 ± 0.4 *	7.5 ± 0.3
HGB	g/dL	14.0 ± 0.4	15.9 ± 0.7 **	15.2 ± 0.9	15.3 ± 0.6 *	14.2 ± 0.3	12.6 ± 0.8 *	14.4 ± 0.8
HCT	%	42.2 ± 1.8	46.3 ± 2.4 *	44.4 ± 2.1	44.5 ± 2.3	42.4 ± 1.2	38.3 ± 2.4 *	42.3 ± 2.7
MCV	fL	56.9 ± 1.0	54.7 ± 1.7	54.2 ± 1.9	54.9 ± 1.9	56.8 ± 1.4	59.2 ± 6.4	56.7 ± 2.4
MCH	pg	19.0 ± 0.3	18.8 ± 0.7	18.5 ± 0.6	18.9 ± 0.6	19.0 ± 0.4	19.6 ± 2.0	19.2 ± 0.7
MCHC	g/dL	33.3 ± 0.4	34.4 ± 0.3 **	34.2 ± 0.5 *	34.3 ± 0.7 **	33.5 ± 0.3	33.0 ± 0.6	33.9 ± 0.3
RET	%	3.2 ± 0.3	0.4 ± 0.1 **	1.9 ± 0.7 **	0.6 ± 0.2 **	3.0 ± 0.5	5.4 ± 0.4 **	2.8 ± 0.5
PLT	$\times 10^3/\mu l$	977.2 ± 127.7	793.6 ± 92.0 *	650.2 ± 140.7 **	757.4 ± 60.6 *	1037.6 ± 67.2	943.6 ± 133.1	1013.0 ± 67.2
WBC	$\times 10^3/\mu l$	6.4 ± 2.3	4.2 ± 2.4	4.3 ± 1.2	2.7 ± 1.3 *	5.3 ± 2.3	4.1 ± 1.0	3.5 ± 1.7
Differential cell count								
NEUT	%	14.7 ± 5.7	13.9 ± 2.9	14.2 ± 5.6	12.7 ± 3.5	13.3 ± 5.1	11.1 ± 2.6	14.5 ± 3.8
LYMPH	%	81.3 ± 6.2	80.7 ± 2.4	81.2 ± 6.3	83.0 ± 4.1	82.6 ± 5.4	83.4 ± 3.8	81.0 ± 4.5
MONO	%	3.0 ± 0.7	3.2 ± 0.7	3.2 ± 0.7	2.4 ± 1.0	3.1 ± 1.0	4.5 ± 1.3	3.1 ± 0.9
EO	%	0.9 ± 0.3	1.9 ± 1.4	1.4 ± 0.8	1.3 ± 0.5	0.9 ± 0.3	0.6 ± 0.1	1.3 ± 0.4
BASO	%	0.1 ± 0.1	0.3 ± 0.3	0.1 ± 0.1	0.6 ± 0.3 *	0.1 ± 0.1	0.4 ± 0.3	0.2 ± 0.2
Female								
No. of animals		5	5	5	5	5	5	5
RBC	$\times 10^6/\mu l$	7.1 ± 0.3	7.1 ± 0.3	7.6 ± 0.6	7.1 ± 0.3	7.1 ± 0.4	6.4 ± 0.7	7.3 ± 0.1
HGB	g/dL	14.0 ± 0.6	13.3 ± 0.4	14.3 ± 1.0	13.5 ± 0.4	13.4 ± 0.9	12.9 ± 1.2	13.8 ± 0.5
HCT	%	40.5 ± 1.6	37.5 ± 0.9	40.8 ± 3.1	38.0 ± 1.1	39.3 ± 2.9	38.5 ± 3.4	40.0 ± 1.4
MCV	fL	56.8 ± 1.5	53.2 ± 1.2 *	53.7 ± 0. 7*	53.9 ± 2.2	55.2 ± 1.4	60.4 ± 2.9 *	54.9 ± 1.7
MCH	pg	19.7 ± 0.7	18.8 ± 0.4	18.9 ± 0.4	19.2 ± 0.7	18.8 ± 0.5	20.1 ± 0.6	18.9 ± 0.4
MCHC	g/dL	34.6 ± 0.3	35.4 ± 0.3 **	35.1 ± 0.4	35.5 ± 0.3 **	34.1 ± 0.3	33.4 ± 0.5 **	34.4 ± 0.5
RET	%	2.5 ± 0.6	0.6 ± 0.3 *	1.9 ± 0.5	0.6 ± 0.2 **	2.4 ± 1.0	4.3 ± 1.8 *	2.1 ± 0.7
PLT	$\times 10^3/\mu l$	993.2 ± 122.4	899.0 ± 183.0	815.0 ± 237.4	656.6 ± 129.7 **	905.6 ± 87.8	881.4 ± 45.3	1035.0 ± 83.7
WBC	$\times 10^3/\mu l$	3.9 ± 2.6	3.2 ± 0.8	3.3 ± 1.7	2.6 ± 0.3	2.7 ± 1.2	3.1 ± 1.7	2.5 ± 0.5
Differential cell count								
NEUT	%	11.9 ± 7.5	7.7 ± 1.7	12.9 ± 3.7	13.1 ± 6.5	8.7 ± 4.7	9.9 ± 4.7	11.9 ± 4.3
LYMPH	%	83.5 ± 8.2	88.2 ± 2.2	82.6 ± 4.2	83.2 ± 7.5	85.5 ± 2.7	84.1 ± 7.4	83.5 ± 4.1
MONO	%	2.8 ± 0.8	2.4 ± 0.7	2.7 ± 0.7	2.2 ± 0.5	3.6 ± 0.4	3.9 ± 1.1	2.7 ± 0.3
EO	%	1.2 ± 0.4	1.4 ± 0.7	1.6 ± 0.8	1.2 ± 0.7	2.1 ± 2.4	1.8 ± 2.1	1.8 ± 0.6
BASO	%	0.6 ± 0.4	0.3 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.1 ± 0.3 *	0.3 ± 0.3	0.1 ± 0.2

Values are mean ± S.D.

*, **: Significantly different from the Basal diet group at $p < 0.05$ and 0.01, respectively.

Table 2. Serum biochemistry data for SD rats

	Basal diet	AMT	VD3	PTU	PB	AGT	E2
Male							
No. of animals	5	5	5	5	5	5	5
TP (g/dL)	5.7 ± 0.2	6.0 ± 0.2	5.8 ± 0.3	6.5 ± 0.2 **	5.8 ± 0.4	6.1 ± 0.4	5.9 ± 0.2
A/G	2.4 ± 0.3	2.7 ± 0.6	2.6 ± 0.3	2.3 ± 0.3	2.1 ± 0.2	2.4 ± 0.3	2.2 ± 0.3
ALB (g/dL)	4.0 ± 0.1	4.4 ± 0.3 *	4.2 ± 0.2	4.5 ± 0.2 **	3.9 ± 0.3	4.3 ± 0.3	4.1 ± 0.1
T-BIL (mg/dL)	0.05 ± 0.01	0.09 ± 0.02 **	0.05 ± 0.01	0.05 ± 0.02	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
GLU (mg/dL)	160.2 ± 21.0	130.6 ± 15.2	185.8 ± 34.2	188.6 ± 34.7	182.0 ± 31.0	169.8 ± 16.2	204.2 ± 45.2
TG (mg/dL)	151.2 ± 68.6	33.4 ± 18.5 **	69.6 ± 17.4 *	59.4 ± 9.6 *	83.2 ± 48.3	45.8 ± 28.5 **	121.8 ± 50.1
T-CHO (mg/dL)	60.6 ± 6.1	134.6 ± 11.4 **	77.8 ± 16.6	99.0 ± 15.9 **	75.6 ± 4.6	89.8 ± 22.8 **	47.8 ± 4.4
BUN (mg/dL)	18.5 ± 1.6	28.0 ± 2.5 **	17.7 ± 2.7	22.8 ± 3.1 *	17.1 ± 2.2	17.8 ± 3.2	16.4 ± 2.1
CRE (mg/dL)	0.26 ± 0.01	0.36 ± 0.02 **	0.23 ± 0.04	0.33 ± 0.03 **	0.25 ± 0.02	0.26 ± 0.03	0.26 ± 0.03
Na (mEq/L)	144.2 ± 1.1	145.2 ± 1.1	144.8 ± 1.3	145.2 ± 1.3	144.4 ± 1.8	145.4 ± 1.1	142.2 ± 2.2
Cl (mEq/L)	104.6 ± 2.4	107.0 ± 1.0	103.0 ± 1.2	105.0 ± 2.5	104.2 ± 2.2	99.8 ± 3.0 **	102.6 ± 1.8
K (mEq/L)	5.1 ± 0.3	4.7 ± 0.2	4.5 ± 0.3 *	4.6 ± 0.1 *	5.1 ± 0.3	4.1 ± 0.6 **	4.7 ± 0.2
Ca (mg/dL)	10.3 ± 0.1	9.8 ± 0.2	14.0 ± 0.7 **	10.2 ± 0.1	10.6 ± 0.2	10.5 ± 0.3	10.2 ± 0.2
IP (mg/dL)	6.9 ± 1.2	5.6 ± 0.5 *	6.7 ± 0.4	5.3 ± 0.4 **	8.1 ± 0.6 *	7.2 ± 0.4	7.5 ± 0.7
AST (IU/L)	118.2 ± 26.8	142.8 ± 23.1	94.8 ± 24.0	99.0 ± 34.9	89.4 ± 15.6	89.8 ± 14.1	74.0 ± 9.0 *
ALT (IU/L)	36.6 ± 2.5	74.8 ± 13.9 **	35.2 ± 5.7	59.8 ± 39.4	38.0 ± 2.9	35.2 ± 4.2	33.0 ± 6.9
ALP (IU/L)	1012.0 ± 62.8	1140.4 ± 201.3	919.6 ± 363.0	674.4 ± 60.0	1043.4 ± 523.1	743.4 ± 119.3	842.6 ± 201.4
γ-GT (IU/L)	<3	4.4 ± 2.2	<3	<3	<3	5.8 ± 2.3 **	<3
Female							
No. of animals	5	5	5	5	5	5	5
TP (g/dL)	6.4 ± 0.2	6.4 ± 0.3	5.6 ± 0.2 **	6.5 ± 0.3	6.1 ± 0.3	6.1 ± 0.5	6.1 ± 0.2
A/G	2.6 ± 0.4	2.2 ± 0.1	2.7 ± 0.4	2.2 ± 0.3	2.5 ± 0.0	2.6 ± 0.3	2.4 ± 0.3
ALB (g/dL)	4.6 ± 0.3	4.4 ± 0.2	4.1 ± 0.2 **	4.4 ± 0.3	4.4 ± 0.2	4.4 ± 0.3	4.3 ± 0.2
T-BIL (mg/dL)	0.04 ± 0.02	0.06 ± 0.01	0.03 ± 0.02	0.04 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.05 ± 0.02
GLU (mg/dL)	177.2 ± 43.4	157.2 ± 33.6	185.2 ± 39.9	190.8 ± 31.8	168.0 ± 24.7	160.6 ± 14.2	191.0 ± 34.9
TG (mg/dL)	94.2 ± 51.2	31.0 ± 13.5 **	37.2 ± 10.4 **	51.6 ± 12.9	61.0 ± 17.4	21.0 ± 7.5 **	58.2 ± 36.2
T-CHO (mg/dL)	69.4 ± 17.8	85.6 ± 7.2	94.2 ± 24.8	81.6 ± 20.2	79.4 ± 9.6	117.0 ± 34.7 **	53.2 ± 7.9
BUN (mg/dL)	19.0 ± 3.2	31.9 ± 5.5 **	15.2 ± 3.7	29.4 ± 1.3 **	17.3 ± 2.5	14.2 ± 2.5	16.4 ± 1.9
CRE (mg/dL)	0.28 ± 0.03	0.42 ± 0.03 **	0.29 ± 0.03	0.42 ± 0.02 **	0.28 ± 0.03	0.25 ± 0.01	0.26 ± 0.01
Na (mEq/L)	142.6 ± 0.5	142.0 ± 1.2	142.6 ± 1.3	142.0 ± 1.0	140.8 ± 1.6	143.4 ± 0.9	139.8 ± 0.8 **
Cl (mEq/L)	102.8 ± 1.8	103.6 ± 1.5	101.8 ± 1.5	103.4 ± 2.3	102.4 ± 0.5	100.0 ± 1.4 *	101.0 ± 1.2
K (mEq/L)	4.5 ± 0.2	4.6 ± 0.2	4.1 ± 0.4	4.2 ± 0.1	4.7 ± 0.5	3.8 ± 0.3 **	4.6 ± 0.3
Ca (mg/dL)	10.3 ± 0.3	10.0 ± 0.5	12.9 ± 0.8 **	9.9 ± 0.4	10.1 ± 0.2	10.4 ± 0.2	10.0 ± 0.3
IP (mg/dL)	6.3 ± 0.9	5.1 ± 0.3 *	6.2 ± 0.5	4.9 ± 0.2 *	6.2 ± 1.0	6.3 ± 0.7	7.0 ± 0.7
AST (IU/L)	107.4 ± 11.7	110.4 ± 11.1	99.2 ± 22.4	84.0 ± 11.1	96.6 ± 18.0	79.6 ± 10.3 *	94.0 ± 8.7
ALT (IU/L)	39.0 ± 7.3	48.2 ± 19.3	38.0 ± 10.2	33.2 ± 1.9	38.4 ± 2.7	34.6 ± 9.1	38.2 ± 3.3
ALP (IU/L)	863.2 ± 201.9	856.4 ± 182.8	808.0 ± 175.9	679.8 ± 281.0	603.6 ± 146.6	573.0 ± 99.2	593.8 ± 192.5
γ-GT (IU/L)	<3	3.2 ± 0.4	<3	<3	<3	7.0 ± 4.7 *	<3

Values are mean ± S.D.

*, **: Significantly different from the Basal diet group at $p < 0.05$ and 0.01, respectively.

Table 3. Serum hormone concentrations for SD rats

	Basal diet	AMT	VD3	PTU	PB	AGT	E2
Male							
No. of animals	5	5	5	5	5	5	5
T3 (ng/mL)	0.9 ± 0.1	0.4 ± 0.0 **	0.9 ± 0.2	0.4 ± 0.0 **	0.9 ± 0.0	0.7 ± 0.2	1.2 ± 0.1 **
T4 (μg/dL)	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 *
TSH (μIU/mL)	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
ACTH (pg/mL)	74.0 ± 36.0	26.0 ± 9.1	43.1 ± 13.5	73.5 ± 71.7	56.8 ± 17.7	216.0 ± 126.6 **	68.1 ± 40.5
FSH (mIU/mL)	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
LH (mIU/mL)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
PRL (ng/mL)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Estradiol (pg/mL)	11.9 ± 3.3	20.5 ± 6.9	11.8 ± 4.0	13.9 ± 1.5	11.6 ± 3.5	20.4 ± 6.3	19.4 ± 11.8
Progesterone (ng/mL)	6.5 ± 3.6	5.1 ± 1.8	3.6 ± 0.9	7.5 ± 3.5	3.0 ± 1.3	5.9 ± 3.0	4.0 ± 1.4
Testosterone (ng/mL)	3.1 ± 2.2	4.5 ± 3.2	3.3 ± 2.2	6.0 ± 8.0	5.1 ± 3.1	4.0 ± 0.9	0.4 ± 0.5
Female							
No. of animals	5	5	5	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/dL)	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
TSH (μIU/mL)	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
ACTH (pg/mL)	56.4 ± 33.3	62.2 ± 40.6	97.2 ± 40.2	48.5 ± 12.5	86.6 ± 48.3	162.6 ± 43.2 **	28.4 ± 10.9
FSH (mIU/mL)	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
LH (mIU/mL)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
PRL (ng/mL)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Estradiol (pg/mL)	20.2 ± 4.9	18.5 ± 5.4	10.4 ± 0.8	22.7 ± 11.4	20.8 ± 9.2	22.1 ± 8.9	21.7 ± 6.9
Progesterone (ng/mL)	16.3 ± 9.0	11.8 ± 6.9	12.9 ± 4.7	23.7 ± 15.3	17.8 ± 14.4	7.4 ± 4.2	7.2 ± 5.2
Testosterone (ng/mL)	<0.03	<0.03	<0.03	0.03 ± 0.01	<0.03	1.71 ± 0.51	<0.03

Values are mean ± S.D.

*, **: Significantly different from the Basal diet group at $p < 0.05$ and 0.01, respectively.

Table 4. Organ weights for SD rats

	Basal diet	AMT	VD3	PTU	PB	AGT	E2
Male							
No. of animals	5	5	5	5	5	5	5
Body weight (g)	386.3 ± 28.2	268.5 ± 2.2 **	278.8 ± 34.7 **	277.5 ± 24.6 **	398.4 ± 9.0	280.6 ± 18.3 **	292.1 ± 4.40 **
Absolute (g)							
Pituitary	0.015 ± 0.003	0.014 ± 0.002	0.011 ± 0.002 **	0.015 ± 0.002	0.014 ± 0.001	0.012 ± 0.002 *	0.013 ± 0.001
Thyroids	0.024 ± 0.002	0.094 ± 0.030 **	0.022 ± 0.004	0.080 ± 0.021 **	0.027 ± 0.004	0.041 ± 0.008	0.025 ± 0.004
Adrenals	0.05 ± 0.00	0.03 ± 0.01 **	0.06 ± 0.01	0.03 ± 0.00 **	0.05 ± 0.00	0.05 ± 0.01	0.06 ± 0.01 *
Liver	14.0 ± 1.3	7.9 ± 0.5 **	9.8 ± 1.7 **	9.3 ± 1.4 **	18.6 ± 1.2 **	15.1 ± 1.0	11.5 ± 0.9 *
Kidneys	2.9 ± 0.3	1.8 ± 0.1 **	2.7 ± 0.2	1.9 ± 0.2 **	3.0 ± 0.1	2.2 ± 0.4 **	2.2 ± 0.3 **
Testes	3.2 ± 0.2	3.0 ± 0.1	2.9 ± 0.1	3.1 ± 0.2	3.2 ± 0.3	3.1 ± 0.3	3.0 ± 0.1
Relative (g%)							
Pituitary	0.004 ± 0.001	0.005 ± 0.001 **	0.004 ± 0.001	0.005 ± 0.001 **	0.004 ± 0.000	0.004 ± 0.001	0.004 ± 0.000
Thyroids	0.006 ± 0.001	0.035 ± 0.011**	0.008 ± 0.001	0.029 ± 0.006 **	0.007 ± 0.001	0.014 ± 0.002	0.009 ± 0.002
Adrenals	0.013 ± 0.001	0.012 ± 0.002	0.021 ± 0.004 **	0.011 ± 0.002	0.014 ± 0.001	0.019 ± 0.003 **	0.021 ± 0.004 **
Liver	3.6 ± 0.2	3.0 ± 0.2 **	3.5 ± 0.2	3.3 ± 0.2	4.7 ± 0.4 **	5.4 ± 0.2 **	3.9 ± 0.3
Kidneys	0.74 ± 0.08	0.67 ± 0.03	0.96 ± 0.12 **	0.68 ± 0.04	0.76 ± 0.03	0.79 ± 0.09	0.77 ± 0.09
Testes	0.84 ± 0.06	1.11 ± 0.06 **	1.06 ± 0.12 **	1.13 ± 0.11 **	0.80 ± 0.06	1.10 ± 0.14 **	1.02 ± 0.04 *
Female							
No. of animals	5	5	5	5	5	5	5
Body weight (g)	248.0 ± 23.0	194.7 ± 11.8 **	180.8 ± 24.6 **	194.8 ± 17.9 **	246.0 ± 22.9	207.6 ± 18.1 *	215.2 ± 16.5
Absolute (g)							
Pituitary	0.017 ± 0.001	0.015 ± 0.002	0.012 ± 0.003 *	0.013 ± 0.003 [b]	0.016 ± 0.003	0.014 ± 0.001	0.014 ± 0.002
Thyroids	0.023 ± 0.003	0.110 ± 0.028 [a]**	0.020 ± 0.006	0.070 ± 0.008 **	0.026 ± 0.004	0.055 ± 0.014 **	0.019 ± 0.004
Adrenals	0.06 ± 0.00	0.03 ± 0.00 **	0.05 ± 0.01	0.03 ± 0.00 **	0.07 ± 0.01	0.06 ± 0.01	0.05 ± 0.01
Liver	9.0 ± 0.5	6.2 ± 0.7 **	6.8 ± 1.0 **	6.8 ± 1.0 **	11.0 ± 1.6 *	11.4 ± 0.9 **	8.3 ± 0.8
Kidneys	1.8 ± 0.1	1.3 ± 0.1 **	2.0 ± 0.3	1.3 ± 0.1 **	1.8 ± 0.1	1.8 ± 0.2	1.7 ± 0.2
Ovaries	0.09 ± 0.02	0.07 ± 0.01	0.06 ± 0.02 *	0.06 ± 0.01	0.09 ± 0.01	0.11 ± 0.02	0.07 ± 0.02
Relative (g%)							
Pituitary	0.007 ± 0.001	0.007 ± 0.001	0.007 ± 0.001	0.007 ± 0.001	0.006 ± 0.001	0.007 ± 0.001	0.007 ± 0.001
Thyroids	0.009 ± 0.001	0.056 ± 0.015 **	0.011 ± 0.003	0.036 ± 0.002 **	0.011 ± 0.002	0.027 ± 0.008 **	0.009 ± 0.002
Adrenals	0.025 ± 0.003	0.017 ± 0.001 **	0.029 ± 0.003 *	0.016 ± 0.002 **	0.027 ± 0.004	0.029 ± 0.002	0.025 ± 0.003
Liver	3.6 ± 0.3	3.2 ± 0.2 *	3.7 ± 0.1	3.5 ± 0.2 **	4.5 ± 0.3 **	5.5 ± 0.2	3.9 ± 0.3
Kidneys	0.74 ± 0.06	0.69 ± 0.04	1.10 ± 0.10 **	0.66 ± 0.03	0.75 ± 0.06	0.85 ± 0.03 *	0.80 ± 0.05
Ovaries	0.035 ± 0.006	0.035 ± 0.006	0.032 ± 0.005	0.033 ± 0.006	0.035 ± 0.003	0.054 ± 0.004 **	0.031 ± 0.011

Each value represents the mean ± S.D. a; n=4, b; n=3.
*, **: Significantly different from the Basal diet group at $p<0.05$ and $p<0.01$, respectively.

Table 5. Histopathological findings for SD rats

	Male							Female						
Treatment	Basal diet	AMT	VD3	PTU	PB	AGT	E2	Basal diet	AMT	VD3	PTU	PB	AGT	E2
No. of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Organ and lesions														
Thyroids														
Hypertrophy, follicular cell	0	5 **	0	5 **	5 **	5 **	0	0	4 [a]**	0	5 **	5 **	5 **	0
Hyperplasia, follicular cell	0	5 **	0	5 **	0	5 **	0	0	4 [a]**	0	5 **	0	5 **	0
Colloid depletion	0	5 **	0	5 **	0	5 **	0	0	4 [a]**	0	5 **	0	5 **	0
Pituitary gland														
Vacuolation, pars distalis	0	5 **	0	5 **	4 *	5 **	0	0	5 **	0	3 [b]*	0	5 **	0
Hypertrophy, pars distalis	0	5 **	0	5 **	4 *	5 **	0	0	5 **	0	3 [b]*	1	5 **	0
Cyst	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Rathke's pouch, persistent	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Adrenal glands														
Vacuolation, cortical, increased, diffuse	0	0	0	0	0	5 **	0	0	0	0	0	0	5 **	0
Hypertrophy, cortical, diffuse	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Atrophy, cortical	0	5 **	0	5 **	0	0	0	0	5 **	0	5 **	0	0	0

*,**; Significantly different from the Basal diet group at $p<0.05$ and 0.01, respectively. a; n=4, b; n=3.

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

平成30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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国立大学法人 東北大学　大学院医学系研究科　教授

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試験を実施中である。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。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、今年度AOPを（Aop:277）を作成した。今回、2019年3月25日国立医薬品食品衛生研究所にて、OECDのAOPプログラムの現在座長でありEuropean Commission, Joint Research Center, ItalyのMaurice Whelan先生から直接作成中のAOPに関して意見を伺うことができ、それを考慮してAOPを修正した。次年度中に、EAGMSTによるreviewを予定している。

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 (H30)

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。今年度、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 を作成した。そこで、MITA の 4 種類のパラメータの内の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。その結果、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。そこで、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 試験を実施中である。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 (H32)

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、Inhibition of IL-1 signalingに関するAOPを作成した (Aop: 277)。AOP WIKIの記載要項に沿って作成した原稿をAppendix 1 AOP for inhibition of IL-1 signalingに示す。また、この内容に関して、OECDのAOPプログラムの現在座長であり European Commission, Joint Research Center, Italy の Maurice Whelan先生のご意見と伺うことができた {2019年3月25日国立医薬品食品衛生研究所}。Whelan先生は、OECDのAOPプログラムの現在座長を務めていられる方で、AOPの作成上の注意点などを直接伺いAOP作成の参考にした。

C.2. Inhibition of IL-1 signaling AOP の AOP WIKI への登録

作成した AOP を AOP WIKI に登録している (AOP 277).

D. 考察

現在、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試験を実施中である。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。

今回，幸いに OECD の AOP 担当座長である Whelan 先生から作成中の AOP に関して意見を伺うことができた。意見は概ね本 AOP に肯定的で、How many Key Events (KEs) are required in the AOP and what is required in Key Event Relationship when the signaling pathway is confirmed?という質問に関して、KEs are prepared for each step, such as macromolecular, cell/Tissue, Organ/Organ system, individual.

Even though the pathway is confirmed, the information regarding empirical evidence, uncertainties and inconsistencies, response-response relationship, time scale should be described.

などの貴重な意見を頂いた。

引用文献

Kimura, Y., Fujimura, C., Ito, Y., Takahashi, T., Terui, H., Aiba, S., 2018. 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. 92, 2043-2054.

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, 1816-1830

E. 結論

Inhibition of IL-1 signalingのAOPを作成し、AOP WIKIに登録中である。また、OECDのAOP担当座長であるWhelan先生から貴重な意見を伺え、それを参考にしてさらなる改善をはかっている。

F. 研究発表

F.1. 論文発表

1. Kimura, Y., Fujimura, C., Ito, Y., Takahashi, T., Terui, H., Aiba, S., 2018a. 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 92, 2043-2054.
2. Kimura, Y., Watanabe, M., Suzuki, N., Iwaki, T., Yamakage, K., Saito, K., Nakajima, Y., Fujimura, C., Ohmiya, Y., Omori, T., Kojima, H., Aiba, S., 2018b. The performance of an in vitro skin sensitisation test, IL-8 Luc assay (OECD442E), and the integrated approach with direct peptide reactive assay (DPRA). J Toxicol Sci 43, 741-749.

F.2. 学会発表

1. 木 村 裕 他 ： Multi-ImmunoTox Assay (MITA) ：バリデーション研究の結果 日本動物実験代替法学会 第31回大会（熊本） 2018年11月

G．知的財産権の出願・登録状況

G.1. 特許取得

なし

H. 添付資料

AOP for inhibition of IL-1 signaling

AOP for inhibition of IL-1 signaling

Table of contents

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-κB is already confirmed. In addition, the biological plausibility that suppressed NF-κB activation leads to impaired T cell activation, resulting in impaired antibody production and that impaired T cell function 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. Beside the blocking of IL-1 binding to its receptor, several drugs also suppress the production of IL-1. Dexamethasone is one of the representatives that significantly suppress IL-1β production from monocytes. Although the effects of dexamethasone are pleiotropic, it is well known to increase the susceptibility to bacterial, fungal, or viral infection. Minocycline or two caspase-1 inhibitors, Pralnacasan (VX-740) and Belnacasan (VX-765, also HMR3480 that are orally absorbed compounds and synthetized as prodrugs which are then converted into the

active principle, VRT-018858 and VRT-043198, respectively also suppress IL-1 signaling by the inhibition of caspase-1 activation, which is an essential enzyme for maturation of pro- IL-1β and the secretion of mature IL-1β. Recently, it has been reported that cinnamicaldehyde suppresses serum IL-1β level in endotoxin poisoning mice. 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).

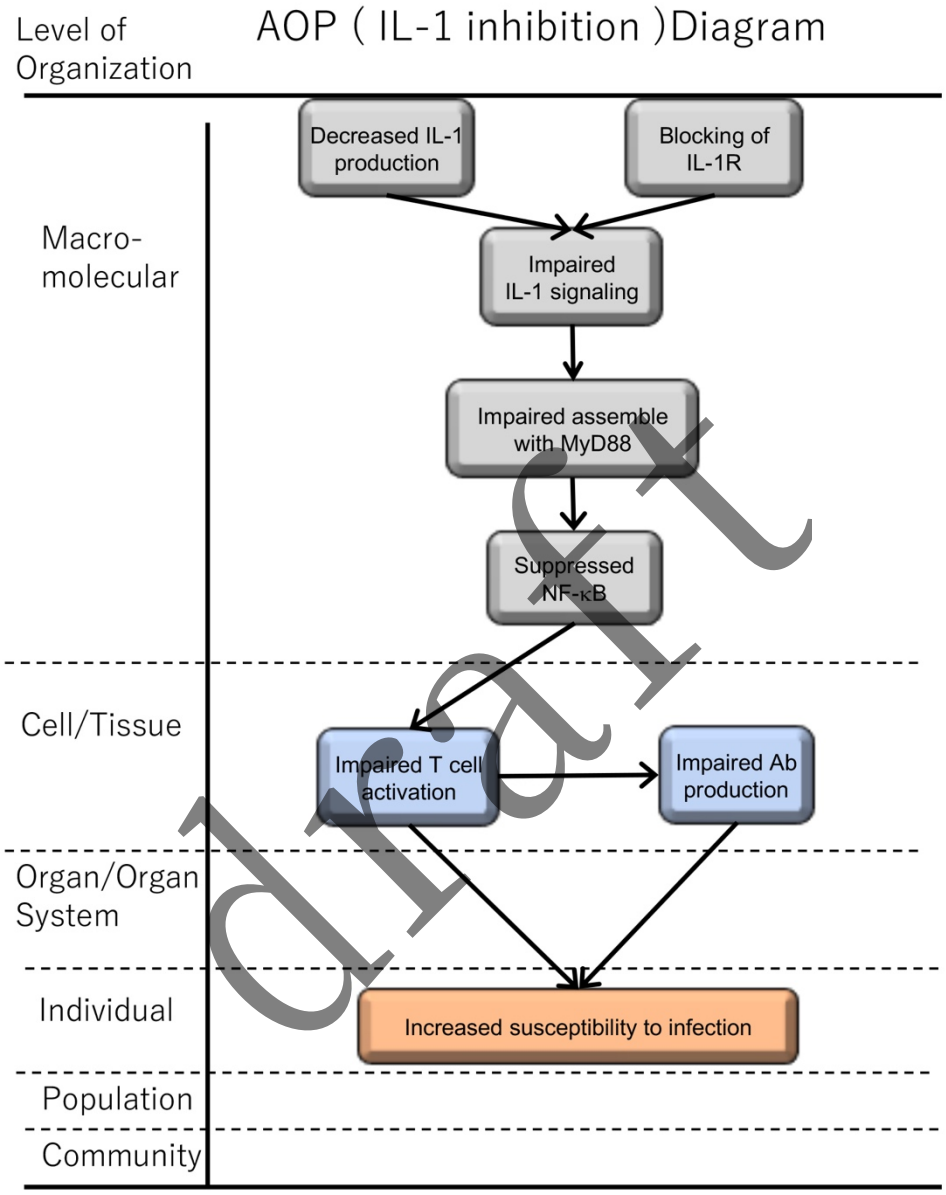
Beside the blocking of IL-1 binding to its receptor, several drugs also suppress the production of IL-1. Dexamethasone is one of the representatives that significantly suppress IL-1β production from monocytes (Finch-Arietta and Cochran, 1991). Minocycline, and pralnacasan (VX-740) and belnacasan (VX-765) that are orally absorbed compounds and synthetized as prodrugs which are then converted into the active principle, VRT-018858 and VRT-043198, respectively (Fenini et al., 2017) also suppress IL-1 signaling by the inhibition of caspase-1 activation, which is an essential enzyme for maturation of pro- IL-1β and the secretion of mature IL-1β(Vincent and Mohr, 2007). Recently, it has been reported that cinnamicaldehyde suppresses serum IL-1β level in endotoxin poisoning mice (Xu et al., 2017). These data suggest that chemicals as well as drugs can suppress IL-1 signaling through their inhibitory effects on IL-1β.

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-κB is already accepted. In addition, the biological plausibility that suppressed NF-κB 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.

Moreover, Patients with defects in MyD88 gene have an increased susceptibility to pyogenic bacterial infections (Picard et al., 2010; von Bernuth et al., 2008)(von Bernuth et al. 2008, Picard et al. 2010). The fact that MyD88 knockout mice showed fatal mycobacterium tuberculosis infection supports the significance of MyD88. (Fremond et al., 2004; Scanga et al., 2004).

These data suggest that IL-1 signaling via MyD88 is indispensable for the defense against microorganisms, and assessment of IL-1 signaling is a good tool for screening the chemical that influence to the host defense.

Summary of the AOP



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

Sequence	Type	Event ID	Title	Short name
	MIE	1570	Blocking of IL-1R	Blocking of IL-1R

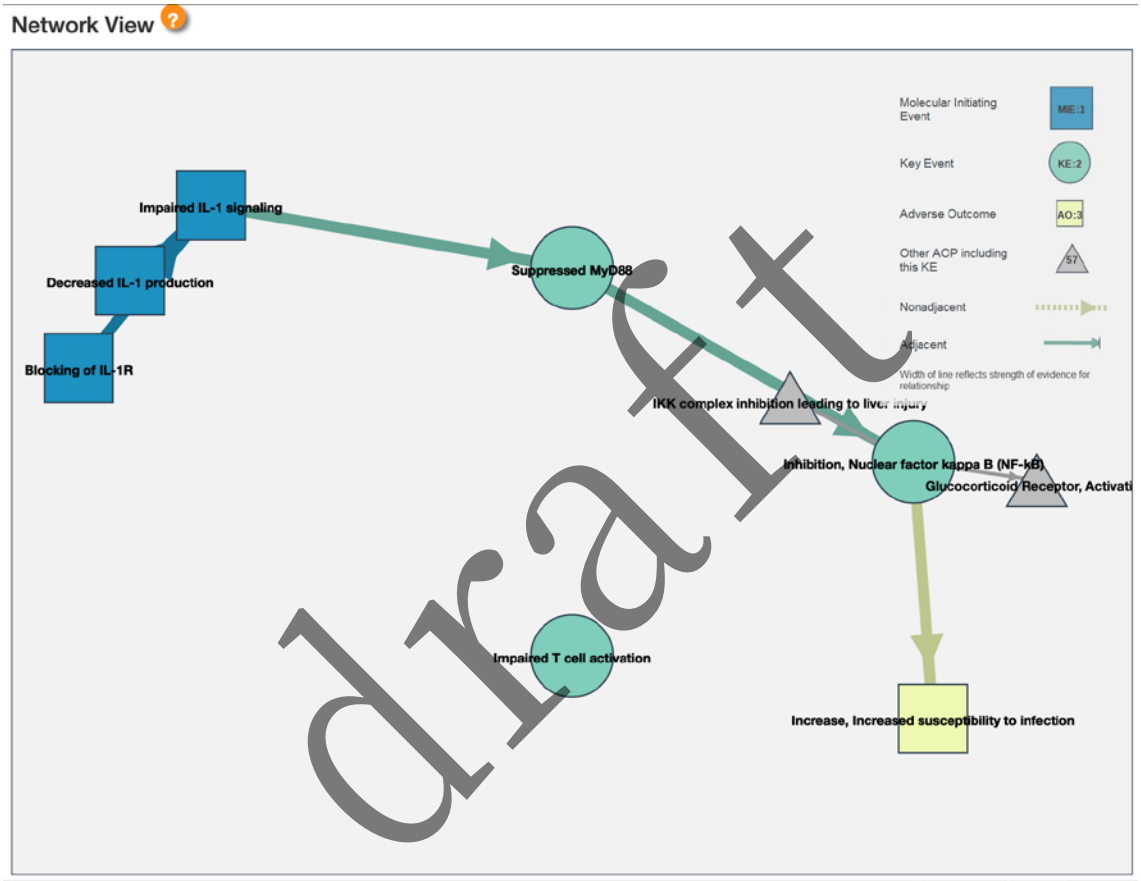
	MIE	1571	Decreased IL-1 production	Decreased IL-1 production
	MIE	1572	Impaired IL-1 signaling	Impaired IL-1 signaling
	KE	202	Inhibition, Nuclear factor kappa B (NF-kB)	Inhibition, Nuclear factor kappa B (NF-kB)
	AO	986	Increase, Increased susceptibility to infection	Increase, Increased susceptibility to infection

Relationships Between Two Key Events (Including MIEs and AOs)

Title	Adjacency	Evidence	Quantitative Understanding
Blocking of IL-1R leads to Impaired IL-1 signaling	adjacent	High	High
Decreased IL-1 production leads to Impaired IL-1 signaling	adjacent	High	High
Impaired IL-1 signaling leads to Suppressed MyD88	adjacent	High	High
Suppressed MyD88 leads to Inhibition, Nuclear factor kappa B (NF-kB)	adjacent	High	High
nhibition, Nuclear factor kappa B (NF-kB) leads to	adjacent	High	High

Increase, Increased susceptibility to infection			

Network View



Stressors

Dexamethosone, minocycline, two caspase-1 inhibitors, Pralnacasan (VX-740) and Belnacasan (VX-765, also HMR3480, cinnamic aldehyde, IL-1 receptor antagonist（IL-1Ra）(Anakinra), anti-IL-1b antibod (Canakinumab), soluble IL-1R (Rilonacept).

Life Stage Applicability
Taxonomic Applicability
Sex Applicability
Overall Assessment of the AOP
Domain of Applicability

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), 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).

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). As IL-1 signaling antagonists, two drugs went up to the market, canakinumab (anti-IL-1b 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).

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. 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-κB p50 are unable effectively to clear L. monocytogenes and are more susceptible to infection with S. peumoniae (Sha et al., 1995).

Evidence Assessment

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).

KER1:Blocking of IL-1R leads to Impaired IL-1 signaling.

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. 1990), IL-6 (Goh et al. 2014), and T cell proliferation (Seckinger et al. 1990).

KER2: Decreased IL-1 production leads to Impaired IL-1 signaling.

Quantitative Understanding

Considerations for Potential Applications of the AOP (optional)

MIE: Title: Inhibition of IL-1 binding to IL-1R

Short name: Inhibition of IL-1 binding to IL-1R

Biological organization: Molecular

Cell term: Macrophage

Organ term: immune system

Stressors

IL-1 receptor antagonist（IL-1Ra）(Anakinra), anti-IL-1b antibody (Canakinumab), soluble IL-1R (Rilonacept)

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-R 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)

Applicability domain

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), 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).

These data suggest that the proposed AOP regarding inhibition of IL-1 signaling is not dependent on life stage, sex, age or species.

Evidence for perturbation of this molecular initiating event by suppressor

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).

MIE: Title: Decreased IL-1 production

Short name: Decreased IL-1 production

Biological organization: Molecular

Cell term: Macrophage

Organ term: immune system

Stressors: Chemical:20384 Dexamethasone, minocycline, pralnacasan (VX-740), belnacasan and cinnamic aldehyde

Key event description

Decreased IL-1 production by macrophages can be induced by suppressed IL-1 b mRNA induction or suppressed maturation of pro-IL-1b which leads to decreased IL-1b secretion. Dexamethasone is one of the representative drugs that significantly suppress IL-1β production from monocytes (Finch-Arietta and Cochran, 1991). Minocycline, and pralnacasan (VX-740) and belnacasan (VX-765) that are orally absorbed compounds and synthetized as prodrugs which are then converted into the active principle, VRT-018858 and VRT-043198, respectively (Fenini et al., 2017) also suppress IL-1 signaling by the inhibition of caspase-1 activation, which is an essential enzyme for maturation of pro- IL-1β and the secretion of mature IL-1β (Vincent and Mohr, 2007).

Recently, it has been reported that cinnamicaldehyde suppresses serum IL-1β level in endotoxin poisoning mice (Xu et al., 2017). These data suggest that chemicals as well as drugs can suppress IL-1 signaling through their inhibitory effects on IL-1β production.

How it is measured or detected.

Inhibition of IL-1 mRNA expression is measured by quantitative real-time polymerase chain reaction.

The production of IL-1b is measured by ELISA (Karpenko et al., 2018).

Applicability domain

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), 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).

These data suggest that the proposed AOP regarding inhibition of IL-1 signaling is not dependent on life stage, sex, age or species.

Evidence for perturbation of this molecular initiating event by stressor

Dexamethasone inhibits IL-1b gene expression in LPS-stimulated RAW 264.7 cells by blocking NF-kB/Rel and AP-1 activation (Jeon et al., 2000).
Dexamethasone suppress LPS-induced gene expression of IL-1 beta in rat lung. (in vivo) (Qiu et al., 1997)
DXM inhibited the release of IL-1b by human leukocyte stimulated with Streptococcus pneumoniae stimulation (van Furth et al., 1995).
Treatment of peripheral blood monocytes with 2 μg/ml lipopolysaccharide potently increased IL-1b release (p= 0.001) and dexamethasone (10^{-7} M) significantly reduced both resting and stimulated IL-Ib release (p 0.009).) (Morand et al., 1993)
DEX a effectively blocks the glutamine antagonist acivicin-induced expression of IL-1b mRNA by HL-60 leukemia cells (Weinberg et al., 1992).

LPS treatment induced a significant upregulation of the mRNA and release of IL-1beta from retinal microglia. Minocycline inhibited its releases. Thus, minocycline might exert its antiinflammatory effect on microglia by inhibiting the expression and release of IL-1beta (Wang et al., 2005).

Caspase-1 inhibition reduced the release of IL-1β in organotypic slices exposed to LPS+ATP. Administration of pralnacasan (intracerebroventricular, 50 μg) or VX-765 (intraperitoneal, 25–200 mg/kg) to rats blocked seizure-induced production of IL-1β in the hippocampus, and resulted in a twofold delay in seizure onset and 50% reduction in seizure duration (Ravizza et al., 2006).
VX-765, an orally active IL-converting enzyme/caspase-1 inhibitor, blocked IL-1b secretion with equal potency in LPS-stimulated cells from FCAS and control subjects (Stack et al., 2005).

The study was intended to examine the protective effect of cinnamaldehyde (CM) on lipopolysaccharide (LPS)-induced acute lung injury (ALI) mice model. The results of the investigation confirmed that, LPS induced inflammatory cytokines such as TNF-α, IL-6, IL-13 and IL-1β were significantly decreased by CM (Huang and Wang, 2017).
The suppressing capacities of six cinnamaldehyde-related compounds were evaluated and compared by using the lipopolysaccharide (LPS)-primed and adenosine 5'-triphosphate (ATP)-activated macrophages. At concentrations of 25~100 μM, cinnamaldehyde and 2-methoxy cinnamaldehyde dose-dependently inhibited IL-1b secretion (Ho et al., 2018).
In vitro, CA decreased the levels of pro-IL-1β and IL-1β in cell culture supernatants, as well as the expression of NLRP3 and IL-1β mRNA in cells. In vivo, CA decreased IL-1β production in serum. Furthermore, CA suppressed LPS-induced NLRP3, p20, Pro-IL-1β, P2X7 receptor (P2X7R) and cathepsin B protein expression in lung, as well as the expression of NLRP3 and IL-1β mRNA (Xu et al., 2017).

KE: Title: Impaired IL-1 signaling

Short name: Impaired IL-1 signaling

Biological organization: Molecular

Cell term: Macrophage

Organ term: immune system

Stressors: Dexamethosone, minocycline, cinnamic aldehyde, IL-1 receptor antagonist（IL-1Ra）(Anakinra), anti-IL-1b antibod (Canakinumab), soluble IL-1R (Rilonacept).

Key event description

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 (Dinarello, 2018; Weber et al., 2018). Therefore, decreased IL-1 production by macrophages, dendritic cells, epithelial cells, and endothelial cells or inhibition of IL-1 binding to IL-1R1 by anti-IL-1β antibody, IL-1RA, or soluble IL-1Ra1 inhibits the formation of the trimeric complex which results in impaired IL-1 signaling.

How it is measured or detected.

It is not possible to directly measure the activation of IL-1 signaling. Instead, the activation of IL-1 signaling can be indirectly measured by the activation of NF-kB or mRNA or protein expression of IL-1 responsive cytokines, such as IL-6 or IL-8, or cyclooxygenase 2.

NFκB p65 (Total/Phospho) ELISA：

ELISA for IL-6, IL-8, and Cox-2.

Applicability domain

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), 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).

These data suggest that the proposed AOP regarding inhibition of IL-1 signaling is not dependent on life stage, sex, age or species.

Evidence for perturbation of this molecular initiating event by stressor

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 reviewed by Gabay et al (Gabay et al., 2010).

Dexamethasone suppression of IL-1 beta-induced cyclooxygenase 2 expression is not mediated by lipocortin-1 in A549 cells (Newman et al., 1994).
Dexamethasone regulates IL-1 beta and TNF-alpha-induced interleukin-8 production in human bone marrow stromal and osteoblast-like cells (Dexamethasone (10^{-7} M) significantly inhibited IL-lb plus TNF-a stimulated IL-8 production in HBMS, MG-63, and hOB cells (Chaudhary and Avioli, 1994).
Dexamethasone blocks the induction of IL-6 and IL-8 by IL-1-stimualted human lung fibroblasts (Monick et al., 1994).

VX-765, an orally active IL-converting enzyme/caspase-1 inhibitor, blocked IL-1b

secretion with equal potency in LPS-stimulated cells from FCAS and control subjects (Stack et al., 2005).

The study was intended to examine the protective effect of cinnamaldehyde (CM) on lipopolysaccharide (LPS)-induced acute lung injury (ALI) mice model. The results of the investigation confirmed that, LPS induced inflammatory cytokines such as TNF-α, IL-6, IL-13 and IL-1β were significantly decreased by CM (Huang and Wang, 2017). Cinnamaldehyde reduced the neurological deficit scores, brain oedema and infarct volume. Cinnamaldehyde suppressed the activation of signal transduction molecules including toll-like receptor 4, tumour necrosis receptor-associated factor 6 and NF-κB, attenuated the increased levels of TNF-α, IL-1β, CCL2 and endothelial-leukocyte adhesion molecule-1 and ultimately reduced leukocyte infiltration into the ischaemic brain areas after cerebral ischaemia (Zhao et al., 2015).

KE: Title: Inhibition, Nuclear factor kappa B (NF-κB)

Short name: Inhibition, Nuclear factor kappa B (NF-κB)

Biological organization: Molecular

Cell term: Macrophage

Organ term: immune system

Stressors:

Key event description

The NF-κB pathway consists of a series of events where the transcription factors of the NF-κB family play the key role. The canonical NF-κB pathway can be activated by a range of stimuli, including TNF receptor activation by TNF-α. Upon pathway activation, the IKK complex will be phosphorylated, which in turn phosphorylates IκBα. This NF-κB inhibitor will be K48-linked ubiquitinated and degradated, allowing NF-κB 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 IκBα and A20. When the NF-κB pathway is inhibited, its translocation will be delayed (or absent), resulting in less or no regulation of NF-κB 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).

How it is measured or detected.

NF-κB transcriptional activity: Beta lactamase reporter gene assay (Miller et al. 2010)

NF-κB transcription: Lentiviral NF-κB FP reporter with flow cytometry (Moujalled et al. 2012)

NF-κB translocation: RelA-GFP reporter assay (Frederiksson 2012) (Huppelschoten 2017)

IκBα phosphorylation: Western blotting (Miller et al. 2010)

NF B p65 (Total/Phospho) ELISA:
ELISA for IL-6, IL-8, and Cox

Applicability domain

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). 17β-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).

Evidence for perturbation of this molecular initiating event by stressor

Dexamethasone inhibits IL-1b gene expression in LPS-stimulated RAW 264.7 cells by blocking NF-kB/Rel and AP-1 activation (Jeon et al., 2000).

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KE: Title: Inhibition, Impaired T cell activation

Short name: Inhibition, Impaired T cell activation

Biological organization: Molecular

Cell term: T cell

Organ term: immune system

Stressors:

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 for solid organ transplantation. 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.

Recognition of MHC/peptide complexes by the TCR and the co-receptors results in T-cell activation (for a review, see [5]). Signalling via the TCR is further supported by co-stimulatory (e.g. CD28) and accessory (e.g. integrins) molecules. Upon TCR ligation, members of the Src family kinases Lck and Fyn phosphorylate the immunoreceptor tyrosine-based signalling motifs (ITAMs) located within the TCR-associated CD3 and ζ chains. This event results in the recruitment of the tyrosine kinase ζ chain–associated protein kinase of 70 kDa (ZAP-70) to the receptor. ZAP-70 is in turn activated and further phosphorylates the linker for activation of T cells (LAT), a transmembrane adaptor molecule that further assembles a complex leading to Ca2+

flux, Ras and protein kinase C (PKC) activation (Figure 1). These events ultimately culminate in gene transcription, proliferation and differentiation of T cells.

T-cell activation and differentiation depends on APCs such as dendritic cells (DCs), macrophages and B cells. Among them, DCs are highly specialized in antigen presentation and in T-cell priming [6]. DCs act as sentinels in the body where they capture antigens. Danger signals such as microbial products or cytokines from injured tissue activate DCs, which in turn migrate to secondary lymphoid organs, where they allow initiation of the immune response [7]. The nature of the stimulus dictates which kind of immune response will be set in motion [8]. Therefore, 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))

In the process of antigen presentation by DCs, macrophages or B cells, T cell activation is impaired.

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

Applicability domain

Evidence for perturbation of this molecular initiating event by stressor
RelB deficient mice had an impaired cellular immunity, as observed in contact sensitivity reaction (Weih et al., 1995).

References

KE: Title: Inhibition, Impaired Ab production

Short name: Inhibition, Impaired Ab production

Biological organization: Molecular

Cell term: B cell

Organ term: immune system

Stressors:

Key event description

ACTIVATION OF B CELLS
Initial encounter of antigen by B-cells occurs in peripheral lymphoid organ where free antigens gain access via lymphatics or are carried by homing dendritic cells, professional antigen-presenting cells from peripheral tissues. The B-cell receptor on B-lymphocytes efficiently captures antigen which is then internalized,processed and returned to the cell surface as peptides bound to Class II MHC molecules. Antigenactivated B-cells then migrate toward the T-cell zones of the lymphoid tissue. Humoral response to most protein antigens requires help from CD4+ T-cells. B-cell–T-cell interaction leads to activation, proliferation and further differentiation of B-cells into plasma cells. Some B-cells migrate from the T-cell zone into a nearby lymphoid follicle where they proliferate and differentiate and establish secondary germinal centers.The rapid proliferation of cells in the germinal centre greatly increases the number of B-cells specific for the pathogen that initiated the antibody response. Furthermore, in the germinal center, somatic hypermutation of immunoglobulin-variable domain genes and affinity maturation occur such that there is a switch from IgM to other isotypes of antibodies and increase in the affinity of antibodies for the inducing antigen. These antigen-activated B-cells then come into contact with specialized stromal cells called follicular dendritic cells that bear unprocessed antigens trapped within the lymphoid follicles. These cells provide survival signals for mature B-lymphocytes that bind cognate antigen on their surface with high affinity. Those B-cells that fail to bind die by apoptosis. Thus, those B-cells that have high-affinity binding to antigens survive the selection process, leave the germinal center to become either memory B-cells or antibody-secreting plasma cells. Plasma cells migrate to the bone marrow and produce the majority of circulating immunoglobins. B-cells that become

memory B-cells reside in the lymphoid organ and can be rapidly activated upon subsequent challenge with the same antigen.

B-CELL–T-CELL INTERACTION

Helper T-cells which recognize antigen on the surface of B-cells become activated and synthesize both cell bound and secreted effector molecules that synergize in B-cell activation (Fig. 1). CD40 ligand (CD40L) is expressed on activated helper T-cells, that binds to CD40 on B-cell surface. Antigen binding and CD40–CD40L interaction provide signals that drive B-cell activation, proliferation and differentiation into plasma cells. Activated B-cells also express other co-stimulatory molecules such as surface B7.1 and B7.2 proteins that bind to CD28 on the surface of T-cells to enhance cognate interaction as well as driving T-cell activation. The B7 molecules are members of the immunoglobulin superfamily that bind to CD28 on naı¨ve T-cells and an additional receptor, CTLA-4 that is expressed on activated T-cells. CTLA-4 binds B7 molecules with higher avidity than CD28 and transduces a negative signal to the activated T-cells in order to limit excessive proliferative response of these activated T-cells. Soluble factors like cytokines are also important inducers of B-cell activation. Interleukin (IL)-4 preferentially induce switching of immunoglobulin isotype to IgG1 and IgE, whereas tissue growth factor (TGF)-b induces switching to IgG2b and IgA. Interferon (IFN)-c induces IgG2a and IgG3 production by activated B-lymphocytes.(reviewed by Mok (Mok, 2010)).

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.

How it is measured or detected.

Ab production can be measured by ELISA.

Applicability domain

Evidence for perturbation of this molecular initiating event by stressor

Mice lacking the p50 subunit of NF-KB show no developmental abnormalities, but exhibit multifocal defects in immune responses involving B lymphocytes and nonspecific responses to infection. B cells do not proliferate in response to bacterial lipopolysaccharide and are defective in basal and specific antibody production. Mice lacking p50 are unable effectively to clear L. monocytogenes and are more susceptible

to infection with S. pneumoniae (Sha et al., 1995).

References

AO: Title: Increased susceptibility to infection

Short name: Increased susceptibility to infection

Biological organization: Individual

Key event description

Complications from infection as a side-effect of administering FK506 was found to be similar to that of cyclosporin A (Ekberg et al. 2007), and recipients of liver transplants treated with FK506 were found to have suffered bacterial, viral, and fungal infections (Alessiani et al. 1991, Fung et al. 1991).

Defect of IL-1 signaling caused by knockout of mice gene or administration of IL-1 receptor antagonist or neutralizing antibodies to human leads to the increased susceptibility to infection. Moreover, polymorphism of IL-1b or IL-1Ra leads to the increased susceptibility to tuberculosis or fungal infection.

Definition

Increased incidence of bacterial infection, tuberculous infection, and viral infection in humans and mice.

How it is measured or detected.

By comparison of the incidence of infection between individuals exposed to stressors and non-exposed individuals.

Applicability domain

The increased susceptibiity to infection caused by IL-1RA or anti-IL-1 antibody has been reported in both humans and mice.

Evidence for perturbation of this molecular initiating event by stressor

Regulatory significance of the adverse outcome

References

Key Event Relationship

Blocking of IL-1R leads to impaired IL-1 signaling

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. Therefore, the suppression of the binding of IL-1 to IL-1R1 suppresses the recruitment of IL-1RacP, which results in impaired IL-1 signaling.

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).

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). As IL-1 signaling antagonists, two drugs went up to the market, canakinumab (anti-IL-1b 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).

Empirical Evidence

IL-1Ra blocks IL-1 signaling:

1) Down modulation of EGF receptor (3 nM of ED50) (Dripps et al., 1991)
2) Suppression of IL-1-induced endothelial cell-leukocyte adhesion (approximately 10 ng/ml of ED50) (Dripps et al., 1991)
3) rhIL-1a-induced mouse thymocytes proliferation (ED50 almost 3 μg/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-lra infusion synthesized significantly less interleukin 6 ex vivo than PBMC from saline-injected controls. (Granowitz et al., 1992)

Canakinumab (ACZ885, Ilaris):

Four patients with active disease each received an i.v. dose of 10 mg/kg canakinumab. relapse. This was possible because IL-1b, which was undetectable in sera of patients at baseline (assay detection limit <0.1 pg/ml), could be detected by an assay that measured IL-1b complexed with antibody. (Lachmann et al. 2009)

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):

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).

Uncertainties and Inconsistencies

Quantitative Understanding of the Linkage

Response-response relationship

IL-1Ra blocks IL-1 signaling:

IL-lra alone at concentrations as high as 1 μg/mL did not induce IL-la, IL-lb, TNFa, or IL-6 synthesis. Suppression of IL-1-induced IL-1, TNFa, or IL-6 synthesis was dose-dependent (P ≦ .0001). At a twofold molar excess, IL-lra inhibited IL-1-induced IL-1 or TNFa synthesis by 50% (P < .01); an equimolar concentration of IL-lra inhibited synthesis of these two cytokines by over 20% (P < .05). A 10-fold molar excess of IL-lra over IL-lb reduced IL-lb-induced IL-la by 95% (P = .01) and IL-la-induced IL-1b by 73% (P < .01). In elutriated monocytes, a 10-fold molar excess of IL-lra reduced IL-lb-induced IL-la by 82% (P < .05), TNFa by 64% (P = .05), and IL-6 by 47% (P < .05). (Granowitz et al., 1992)

Canakinumab (ACZ885, Ilaris):

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):
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).

Time-scale
Known modulating factors
Known Feedforward/Feedback loops influencing this KER
Domain of Applicability

Decreased IL-1 production leads to impaired IL-1 signaling

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.

Evidence Supporting this KER

Biological plausibility

Empirical Evidence

Uncertainties and Inconsistencies

Quantitative Understanding of the Linkage

Response-response relationship

Time-scale

Known modulating factors

Known Feedforward/Feedback loops influencing this KER

Domain of Applicability

Impaired IL-1 signaling leads to inhibition, Nuclear facto kappa B (NF-kB)

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 (Cavalli et al., 2015). Through conserved cytosolic regions called Toll- and IL-1R–like (TIR) domains (Radons et al., 2003), 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 (Brikos et al., 2007; Li et al., 2002). Mice lacking MYD88 or IRAK4 show severe defects in IL-1 signaling (Adachi et al., 1998; Medzhitov et al., 1998; Suzuki et al., 2002). Similarly, humans with mutations in the IRAK4 gene have defects in IL-1RI and Toll-like receptor (TLR) signaling (Picard et al., 2003). 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. (Brikos et al., 2007).

Evidence Supporting this KER

Biological plausibility

Empirical Evidence

Uncertainties and Inconsistencies

Quantitative Understanding of the Linkage

Response-response relationship

Time-scale

Known modulating factors

Known Feedforward/Feedback loops influencing this KER

Domain of Applicability

Inhibition, Nuclear facto kappa B (NF-κB) leads to impaired T cell activation
Key Event Relationship Description

The general consensus understanding is that 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). Phosphory- lated CD3 activates the T cell specific tyrosine kinase, zeta-chain associated protein kinase (ZAP-70), which phosphorylates the adapter proteins linker for activation of T cells (LAT) and SH2 domain containing leukocyte protein of 76 kDa (SLP-76), causing SLP-76 to bind to VAV1. The VAV1–SLP76–IL-2-inducible T cell kinase (ITK) complex activates phospholipase (PL)C*g*1, generat- ing inositol 1,4,5-tripohosphate (IP3) and diacylglycerol (DAG), which ultimately trigger calcium release and pro- tein 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 ulti- mately 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). This molecular interaction activates phosphoinositide 3-kinase (PI3K), inducing triggers a conformational change, causing CARMA1 to bind to B cell leukemia/lymphoma (BCL10) and mucosa- associated lymphoid tissue lymphoma translocation pro- tein (MALT1), forming the CARMA1-BCL10-MALT1 (CBM) complex. Through a mechanism that may involve TNF receptor-associated factor (TRAF6), both BCL10 and MALT1 become polyubiquitinated. The I*k*B kinase (IKK) complex is then recruited to the CBM complex via the IKK*g* polyubiquitin binding motif. This association leads to polyubiquitination of IKK*g* and phosphorylation of IKK*b* by TGF-*b* activated kinase (TAK1), activating IKK*b*. IKK*b* then phosphorylates inhibitor of *k*B (I*k*B*a*), triggering its proteasomal degradation, enabling nuclear translocation of canonical NF-*k*B heterodimers comprised of p65 reticuloendotheliosis viral oncogene homolog A (RELA) and p50 proteins. 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

RelB deficient mice had an impaired cellular immunity, as observed in contact sensitivity reaction (Weih et al., 1995).

Empirical Evidence

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).

In Balb/c mice (p.o.) treated with naringin (20, 40 and 80 mg/kg) for 14 days, compared with the vehicle-treated and arthritic-control mice, the naringin treatment demonstrated a considerable decrease in the level of T cells, CD4+GITR+, Th1 cytokine and inflammatory mediator expressions. In contrast, naringin treatment resulted in significantly up-regulated Treg and Th2 cytokine levels (Ahmad et al., 2014).

Uncertainties and Inconsistencies

Quantitative Understanding of the Linkage

Response-response relationship

Time-scale

Known modulating factors

Known Feedforward/Feedback loops influencing this KER

Domain of Applicability

Impaired T cell activation to Impaired Ab production

Key Event Relationship Description

'Help' to B cells is not a single product of TFH cells and not even a single process. T cell help to B cells can be divided into seven distinct functions, proliferation, survival, plasma cell differentiation, somatic hypermutation, class-switch recombination, adhesion and attraction. These seven different forms of help are all contributors to T_{FH} cell–B cell interactions, and each process consists of multiple pathways. Furthermore, some molecules have a role in several different forms of help.

The simplest B cell help function that is provided by T_{FH} cells is the induction of B cell proliferation. CD40L is the most prominent protein expressed by T_{FH} cells that contributes to pro-mitotic signalling in B cells64. Survival signals from TFH cells are also crucial, as germinal centre B cells are exquisitely pro-apoptotic. IL-4 produced by T_{FH} cells triggers pro-survival signals to germinal centre B cells via the IL-4 receptor complex. Somatic hypermutation is central to germinal centre biology and the primary purpose of germinal centres is to facilitate affinity maturation of B cells via sequential rounds of immunoglobulin gene mutation and selection. The enzyme activation-induced cytidine deaminase (AID) induces the DNA damage in the immunoglobulin genes that is then converted into mutations by DNA repair enzymes. BCL-6 must be co-expressed with AID by the germinal centre B cell to repress the DNA damage response programme that would otherwise trigger self-destruction of the cell. The signals that induce AID and BCL-6 expression by B cells are not entirely defined, but CD40L, IL-4 and IL-21 contribute. Indeed, the combination of CD40L, IL-4 and IL-21 in different ratios seems to be the primary mix of T cell help signals that control B cell proliferation, somatic hypermutation and differentiation. Class-switch recombination can also be induced by instructive signals from T_{FH} cells to B cells. AID is necessary for class-switch recombination, but the specific target of the heavy chain constant region gene recombination depends on additional factors that are selectively activated by different cytokines, which predominantly, but not exclusively, come from CD4+ T cells. Human IgM to IgG class-switch recombination is most efficiently induced by IL-21, whereas IgE recombination is induced by a high IL-4 to IL-21 ratio.

B cell help crucially depends on cell contact, probably because of a mixture of cell-surface co-stimulatory ligand interactions and directional cytokine production during cognate interactions. Therefore, adhesion molecules expressed by T_{FH} cells and B cells are necessary components of T cell help to B cells, as they regulate the overall duration of the 'pas de deux'. The most dramatic example of this requirement is SAP, which is

described above. SLAM-associated protein (SAP; also known as SH2D1A) binds to the intracellular domains of SLAM family surface receptors, which are involved in cell–cell adhesion. In the absence of SAP, the duration of B cell–T cell adhesion is short and inadequate for the T_{FH} cell to provide sufficient help signals to the B cell. This leads to a general defect in SAP-dependent T cell help to B cells and thus a loss of antigen-specific B cell proliferation and survival, as well as a complete loss of germinal centres and of most memory B cells and long-lived plasma cells.
Finally, chemoattraction is another component of T cell help to B cells. CXC-chemokine ligand 13 (CXCL13) is the ligand for CXCR5 and human germinal centre TFH cells constitutively secrete copious quantities of CXCL13, which probably recruits B cells to colocalize with the TFH cells and to facilitate confinement of the B cells to the germinal centre. Notably, CXCL13 signalling via CXCR5 also modifies B cell adhesion and lymphotoxin synthesis, which shows that CXCL13 also has cytokine-type functions. Thus, chemoattraction is another form of T cell help to B cells.
Therefore, it is conceivable that impaired T cell activation leads to impaired B cell activation and antibody production.

Evidence Supporting this KER
Biological plausibility
Empirical Evidence
Uncertainties and Inconsistencies
Quantitative Understanding of the Linkage
Response-response relationship
Time-scale
Known modulating factors
Known Feedforward/Feedback loops influencing this KER
Domain of Applicability

Impaired T cell activation and Ab production to increased susceptibility to infection

Key Event Relationship Description

Normal T cell and B cell function is indispensable for host defense mechanism.

Evidence Supporting this KER

Biological plausibility

SCID mice and patients with severe combined immunodeficiency are extremely susceptible to bacterial and viral infection.

Empirical Evidence

Uncertainties and Inconsistencies

Quantitative Understanding of the Linkage

Response-response relationship

Time-scale

Known modulating factors

Known Feedforward/Feedback loops influencing this KER

Domain of Applicability

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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

平成30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。そこで、NGTxC・IATA の expert working group として、Bhas42CTA における非遺伝毒性発がん性発現の機序解析データを NGTxC・IATA に提供したところ、Simplified NGTxC AOP flow」が組み立てられ、NGTxC・IATA における有害性発現経路（AOP: Adverse Outcome Pathway）の構築と Bhas42CTA の Test Guideline（TG）開発に繋がり得る活動となった。

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、Bhas42CTAにおける非遺伝毒性発がん性発現の機序解析データをNGTxC・IATAに提供することにより、Bhas42CTAのTG開発に寄与する。

B. 研究方法

OECDのNGTxC・IATAの専門家メンバー間で実施された電話会議等での打ち合わせを行うとともに、IATAに関わるAssay templateの作成を行った。2018年6月には、OECDにて開催された専門家会議に参加し、Assay template として提出したBhas42細胞形質転換試験法（Bhas42CTA）の研究背景と研究状況について報告するとともに、NGTxC・IATAの構築に向けた調整を行った。また、TG及びAOP作成に資する情報を得るため、OECDの分子スクリーニングとトキシコゲノミクスに関する専門家会議に参加した。

（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の専門家メンバー間で実施された電話会議等では、6月の

OECDでの対面会議に向けての調整およびAssay templateの作成についての説明が行われた。日本に対しては、Bhas42CTAのプロモーション試験についてAssay templateの作成が依頼されたため、Bhas 42 Cell transformation assay template（Promotion test））を提出した。6月の対面会議では、非遺伝毒性発がん物質の検出法に関わる情報提供として、「Bhas 42 cell transformation assay (Bhas 42 CTA)」のプレゼンテーションを行い、Bhas42CTA研究の背景と機序解析の進捗状況を報告した。その結果、複数のKey Eventで構成された「Simplified NGTxC AOP flow」が組み立てられ、各Key Eventに関わる試験法の取りまとめを行うため、13ブロックのreviewing teamsを各専門家メンバーが分担した。6月の対面会議後にも電話会議で調整を行い、各試験法のAssay template作成法および評価法についての調整を行った。

また、IATA構築やTG開発に関連するAOP作成の情報を得るため、6月にOECDでの分子スクリーニングとトキシコゲノミクスに関する専門家会議に参加し、NGTxCのAOP作成に資する情報を得ることができた。

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の専門家会議の活動および分子スクリーニングとトキシコゲノミクスに関する専門家会議への参加は、NGTxC・IATAにおけるAOPの構築とBhas42CTA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

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

H. 添付資料

Bhas 42 cell transformation assay

Bhas 42 CTA

添付資料

Bhas 42 cell transformation assay
Bhas 42 CTA

Kiyomi Ohmori
Kanagawa Prefectural Institute of Public Health

Bhas 42 Cell Transformation Assay (Bhas 42 CTA)

Development of “Bhas Promotion Assay; cell transformation assay for the detection of non-genotoxic carcinogen using Bhas 42 cells* ”
(in 2000 by research grants of Kanagawa Prefecture)

* derived as a clone formed by the stable transfection of the v-Ha-ras oncogene into the BALB/3T3 A31-1-1 established by Sasaki *et al.* in 1989

↓

An Inter-laboratory Collaborative Study by the Non-Genotoxic Carcinogen Study Group in Japan, on “Bhas Promotion Assay”
(from 2001 to 2004 by research grants of Japan Chemical Industry Association)

↓

Framework of the protocol on Bhas 42 CTA

↓

Added an initiation test by Food and Drug Safety Center (in 2005)

↓

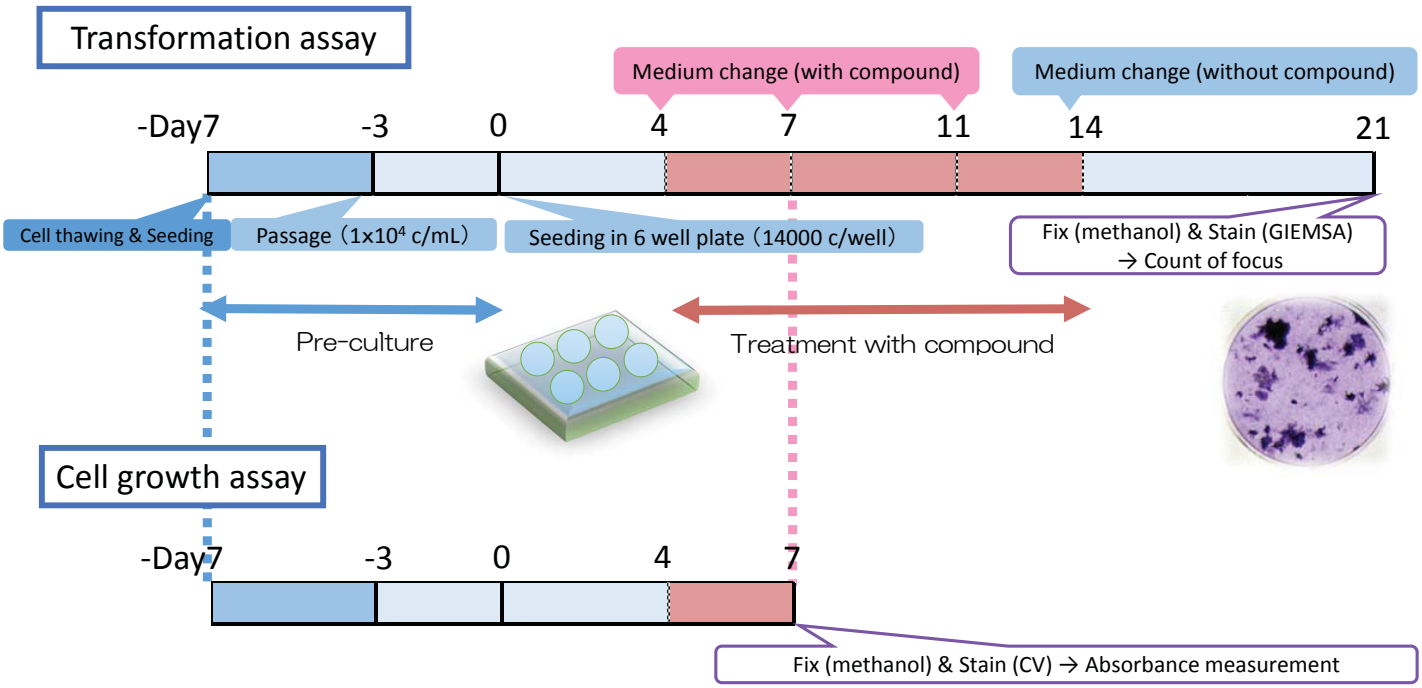
New proposal to OECD by Ministry of Economy, Trade and Industry in Japan and JaCVAM

↓

EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)
EURL ECVAM RECOMMENDATION on the Cell Transformation Assay based on the Bhas 42 cell line
Nov-2013

GUIDANCE DOCUMENT ON THE IN VITRO BHAS 42 CELL TRANSFORMATION ASSAY
Series on Testing & Assessment No. 231
08-Jan-2016, revised 20-Jul-2017

Bhas 42 Cell Transformation Assay (Bhas 42 CTA) for the detection of NGTxC



Advantage of Bhas 42 Cell Transformation Assay (Bhas 42 CTA)

Bhas 42 CTA does not require special facilities, equipment and technology.

Strengths of Bhas 42 CTA

(1) a phenotypic anchoring of onco-transformation.

(2) treatment with an initiating agent and subsequent cultivation for the expression period can be omitted.

(3) the latency period of responsiveness is relatively brief: the experimental period with the focus formation is shortened from 4-6 weeks to 3 weeks after cell inoculation.

(4) Bhas 42 cells are especially responsive to chemical carcinogens and are readily transformed by such agents, resulting in relatively high transformation frequencies

(5) the number of culture vessels necessary for a given assessment of a chemical's non-genotoxic carcinogenic potential is reduced; formation frequency is high, so that only six wells of 6-well plates in transformation assay.

Further studies on Bhas 42 CTA

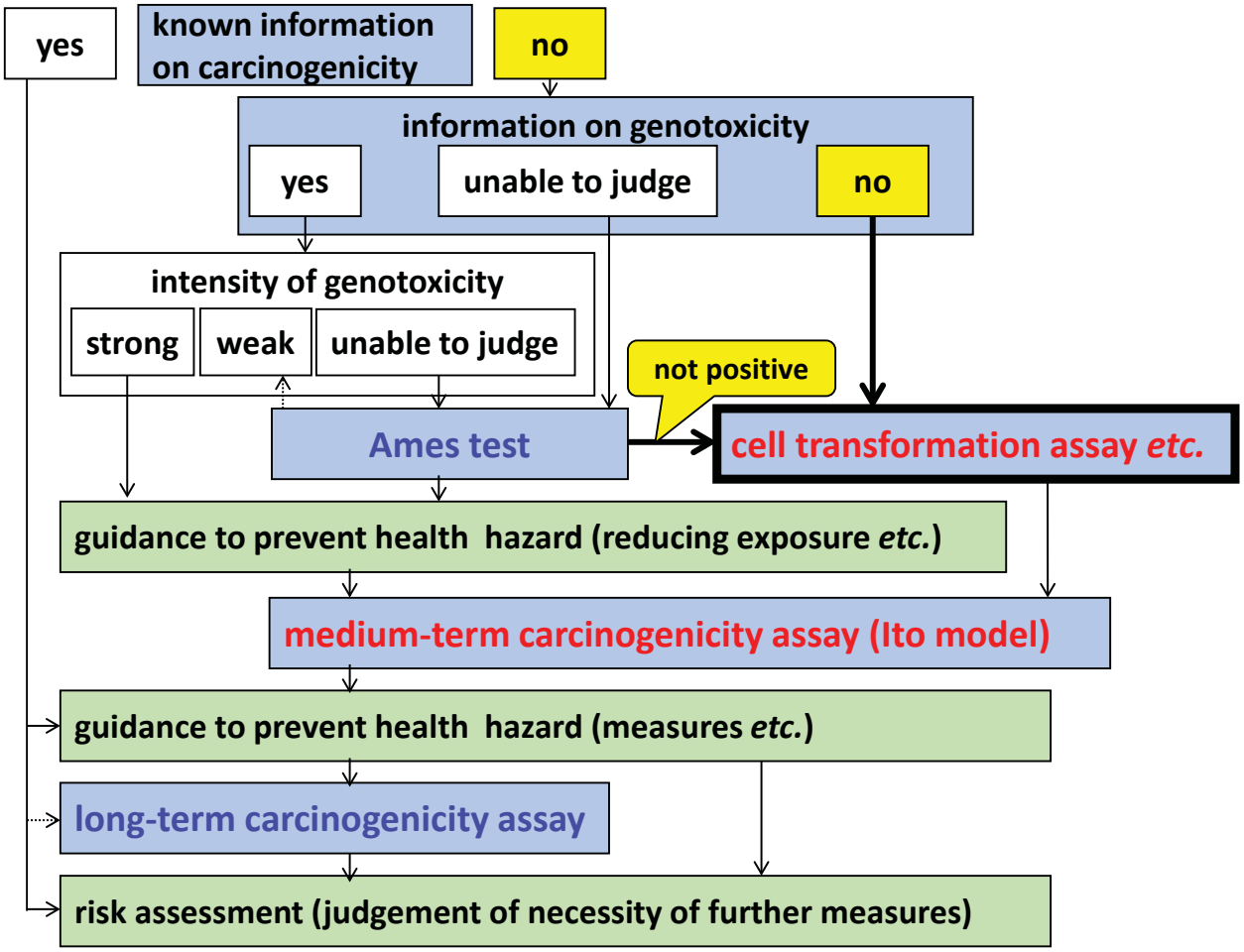
Analysis	Method	Sample	Status
Transcriptomics analysis	Affymetrix mouse Genome 430 2.0 Array	TPA	Almost ready for submission
Transcriptomics analysis	nAnT-iCAGE	23 Compounds	Under analysis
Phosphorylated proteomics analysis	titania-beads method and LC/MS/MS	8 Compounds	Under analysis
Metabolism in Bhas 42 CTA	Expression and activity of CYP enzymes	3-methyl corantherene	Preparation for submission
Metabolism in Bhas 42 CTA	Co-culture with human hepatocyte		Under development

Metabolism of Bhas 42 CTA

- Analysis of CYP enzymes in Bhas 42 cells
  - Expression of the protein
  - Activity of the enzyme

- Development of the Bhas 42 CTA added with the metabolic system in human hepatocyte

Flowchart of carcinogenicity assessment of work place chemical by MHLW



Collaborative research institution and Grants

Research grants of Kanagawa Prefecture

- Transcriptomics analysis by microarray analysis
  Kanagawa Institute of Industrial Science and Technology
- Transcriptomics analysis by nAnT-iCAGE method
  RIKEN Yokohama
- Phosphorylated proteomics analysis using LC/MS/MS
  Yokohama City University

Research grants of Ministry of Education, Culture, Sports, Science and Technology

- Metabolism in Bhas 42 CTA
  Kanagawa Institute of Industrial Science and Technology

Research grants of Ministry of Health, Labor and Welfare

- Cooperation in the AOP construction of NGTxC using Bhas42CTA
  National Institute of Health Sciences

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

平成 30 年度 分担研究報告書

光安全性試験の TG および光毒性 AOP の開発

研究分担者　尾上 誠良　静岡県立大学　薬学部　薬剤学分野　教授

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した OECD test guideline 化を進めており、既にパブリックコメントに対応した TG 案改定を実施した。

研究協力者
世戸 孝樹（静岡県立大学 薬学部 講師）

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被験物質の *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。AOP については AOP wiki に入力し、OECD TG については 2 度目のパブリックコメントに対応して改定案を作成した。改定案については OECD における expert meeting にて紹介・説明し、ほぼ了承された。

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, 6 種の光毒性化合物 [acridine (ACD), furosemide (FSM), hexachlorophene (HCP), 8-methoxypsoralen (MOP), norfloxacin (NFX), promethazine (PMZ)] について ROS assay を行った。

B.2. *In vitro* 皮膚内動態実験

上記 6 種の光毒性化合物について、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。

ドナー側に被験物質 (各 1 mg/mL) を入れ、経時的に皮膚を透過した被験物質量を 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 *in vitro* 皮膚透過性のデータを基に定常状態における各被験物質の皮膚内濃度 (C_{ss}) を算出した。得られたデータと光化学的特性データを併せて考慮することで *in vitro* 光毒性予測を実施した。

B.3. ラット *in vivo* 光毒性試験

腹部を剃毛したラットに被験物質 (10 mg/site) を塗布し、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 種の光毒性化合物 (ACD, FSM, HCP, MOP, NFX および PMZ) は露光時に光安全性評価における 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 ACD は $^1O_2$ およびO_2^- ならびに HCP は O_2^- において他の被験物質と比し強い ROS 産生を示した。*In vitro* 皮膚透過性を基に算出した C_{ss} は ACD, HCP および PMZ がそれぞれ 69.1, 57.3 および 59.2 μg/mL と高く、次に MOP が 50.1 μg/mL と高値を示した。FSM および NFX の C_{ss} の値はそれぞれ 2.8 および 3.2 μg/mL と低値を示した。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：

ACD＞HCP＞PMZ＞MOP＞FSM≒NFX

ラット *in vivo* 皮膚光毒性について 6 種の光毒性化合物は全て陽性と判断し、*in vivo* 光毒性の強さは以下の順であった。

In vivo 光毒性：

ACD≒HCP＞PMZ＞MOP＞FSM＞NFX

C.2. AOP および OECD TG 案の作成

化学物質の光化学的反応を中心とした光毒性に関する AOP を作成し、AOP wiki に入力した。また、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（2 回目）に対応し、TG 改定案を作成した。2018 年 11 月の OECD における専門家会議にて改訂内容について説明し、概ね同意を得ることが出来た。2019 年度には本 TG 案が承認されるものと考える。

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 *in vitro* 光毒性予測系を構築し、6 種の光毒性化合物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 *in vitro* 光安全性評価系を構築し、光化学的特性ならびに皮膚内動態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現在提案中の光毒性に関す

る AOP および ROS assay の OECD TG 化の実現に大きく貢献できると期待する。

F. 研究発表

F.1. 論文発表

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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

H. 添付資料

AOP WiKi

Reactive oxygen species generated form photoreactive chemicals leading to phototoxic reactions

AOP Title

Reactive oxygen species generated form photoreactive chemicals leading to phototoxic reactions

Short name

ROS-mediated chemical phototoxicity

Graphical Representation

AOP diagram (PPT file)

Abstract

Phototoxicity is an adverse reaction in the light-exposed tissues triggered by normally harmless doses of sunlight (Moore, 1998, 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.

Background (Optional)

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	ROS generation
	KE	1594	Oxidation of membrane lipids	Oxidation of membrane lipids
	KE	1595	Oxidation/denatuation of membrane proteins	Oxidation/denatuation of membrane proteins
	AO	1599	Inflamatory events in light-exposed tissues	Inflammatory events

Relationships Between Two Key Events (Including MIEs and AOs)

Title	Adjacency	Evidence	Quantitative Understanding
ROS generation leads to Oxidation of membrane lipids	adjacent	High	Low
ROS generation leads to Oxidation/denatuation of membrane proteins	adjacent	High	Low
Oxidation of membrane lipids leads to Inflammatory events	adjacent	High	Low
Oxidation/denatuation of membrane proteins leads to Inflammatory events	adjacent	High	Low

Stressors

Sunlight (wavelength: 290-700 nm) High
Photoreactive chemicals High
Reactive oxygen species High

Life Stage Applicability

All life stages High

Taxonomic Applicability

Human Homo sapience High

Sex Applicability

Mixed 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, 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

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) in ICH S10 guideline for photosafety assessment (ICH, 2014).

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".

Evidence Assessment

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, 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	Biological Plausibility of the KE 2 => AO is high.

	inflammatory event.	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, 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, 2002, Roberts, 2001).

Empirical Support for KERs

MIE => KE 1: ROS generation leads to Oxidation of membrane lipids	Empirical support of the MIE 2=> KE 1 is strong. Rationale: Lipid peroxidation was occurred by ROS-generated 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 2=> 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 understanding

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 combine with assay data based on AOP.

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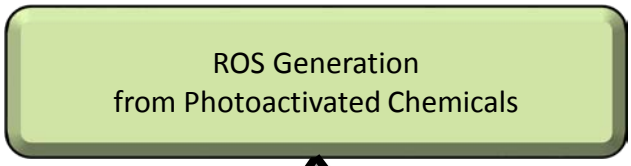
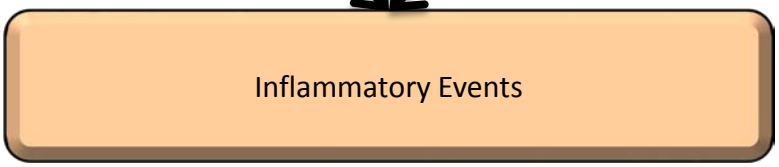
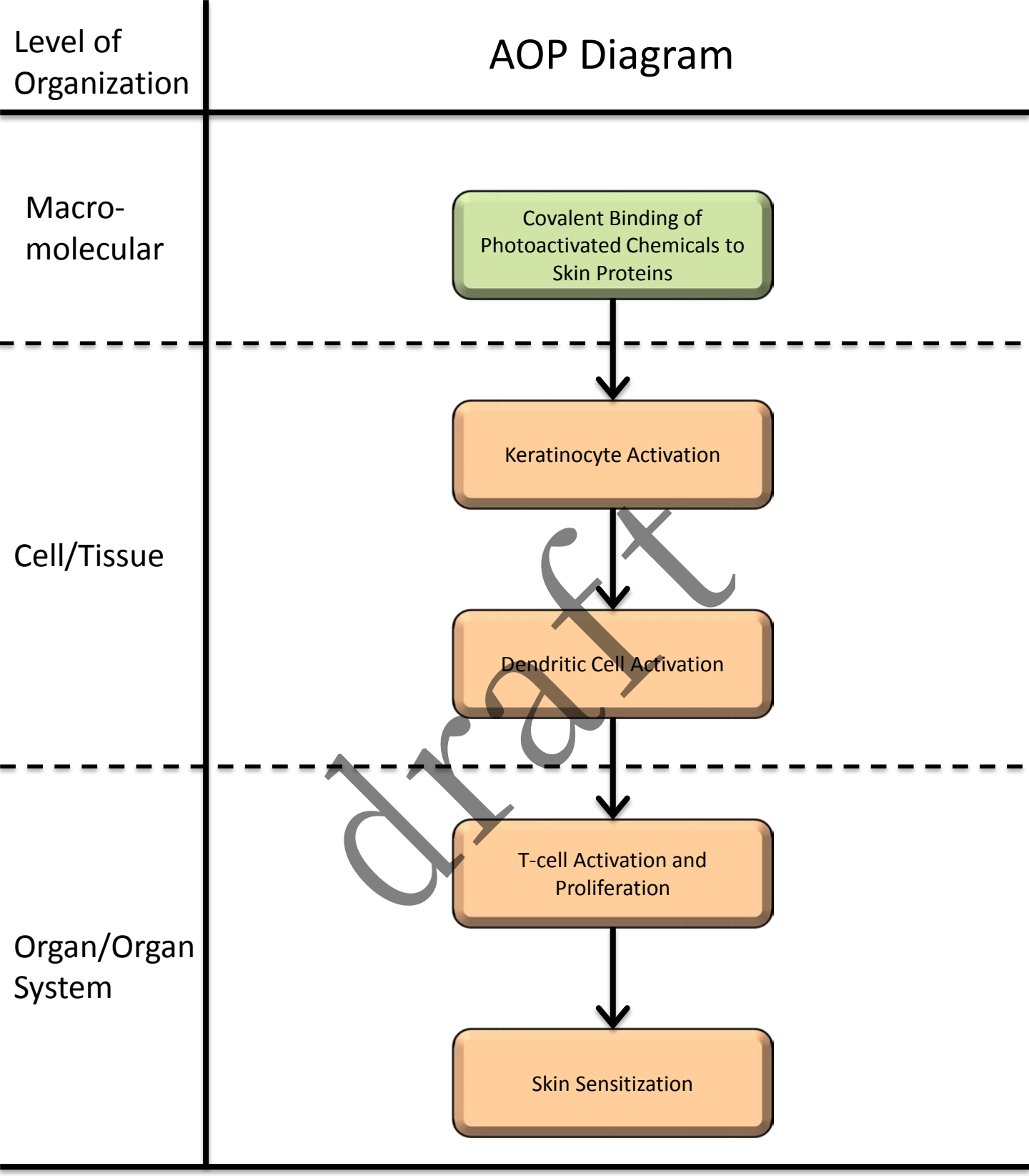
Level of Organization	AOP Diagram
Macro-molecular	
Cell/Tissue	
Organ/Organ System	


Level of Organization
AOP Diagram
Macro-molecular
Cell/Tissue
Organ/Organ System
Covalent Binding of Photoactivated Chemicals to Skin Proteins
Keratinocyte Activation
Dendritic Cell Activation
T-cell Activation and Proliferation
Skin Sensitization

Instructions

- Select a template slide
- Edit the template as needed to provide the specific details of the AOP you're describing
- While viewing the slide you have edited, click Save As – Other formats (fig1)
- In the save dialog box, select "JPEG File Interchange Format" (fig2)
- In the next box, click "Current Slide Only" (fig3)

Fig 1

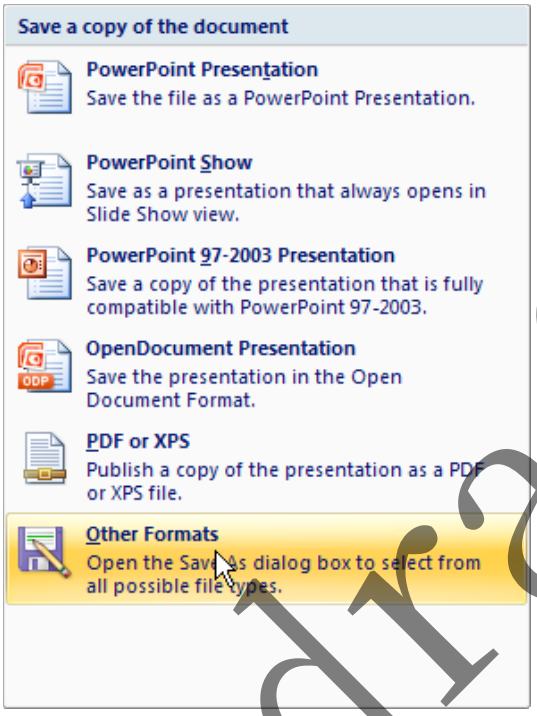


Fig 2

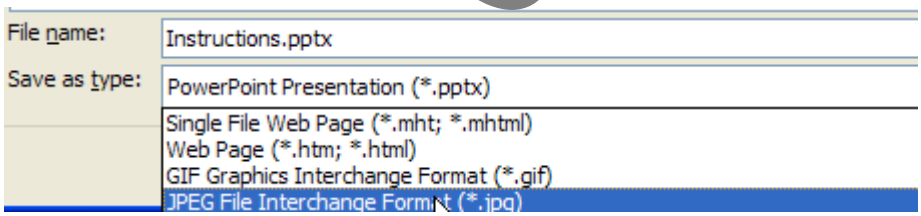
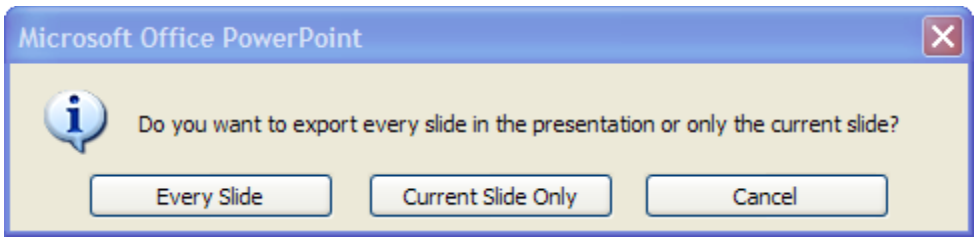


Fig 3



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

平成30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AOPの開発を行った。昨年度AOP wiki登録を行った「Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response」については、OECD EAGMSTより提出された内部レビューアーのマイナーコメントに対応し、内部レビューが終了した。本AOPについては今後外部レビューに進む予定である。

今年度はさらに、以下に示す新たな3種のAOPの開発を行い、SPSF（Standard Project Submission Form）をOECDに提出した。

1. Activation of TLR 7 leading to psoriatic skin disease
2. Activation of estrogen receptors in T cells leading to development of Th2
3. Inhibition of JAK3 leading to impairment of TDAR

これらの新たなAOPについて、次年度AOP wiki登録を行うために、登録書類の原稿案を作成した。

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

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に関するAOPの開発を委託した。

（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

AOP wikiへの登録を行った「Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response」については、2018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、内部レビューの結果が報告された。本AOP

は、FK506などの化学物質がカルシニューリンを阻害することによりIL-2およびIL-4の産生が抑制され、最終的に免疫応答としての抗体産生が阻害されるというものである。会議の結果、本AOPの完成度は高く、内部レビューにおいて特に大きな問題はないため、外部レビューに進むべきとの結論となった。ただし申請したAO (Adverse Outcome) であるTDAR (T cell Dependent Antibody Response)はいわゆるバイオマーカーであり、AOではないとの意見が出されたため、会議後にこの点についてAOP検討小委員会で対応を検討した。当初本AOPでは、AOとして易感染性を提案しており、それに戻すという考えもあったが、TDARは規制側が評価可能なエンドポイント(ICH-S8)であることから、このまま外部レビューに進行することとした。すでに内部レビューアーのマイナーコメントに対応済みであり、今後外部レビューを受ける予定である。

本研究では、さらに免疫毒性のAOPを開発することとし、最終的に以下の３つのAOP案を策定した。１つ目は、「Activation of TLR 7 leading to psoriatic skin disease」というタイトルであり、樹状細胞に存在するTLR (Toll-like receptor) 7の過剰な活性化が、Th17およびKeratinocyteの関与を経て乾癬様の皮膚疾患を生じさせるという内容である。２つ目は、「Activation of estrogen receptors in T cells leading to development of Th2」というタイトルで、T細胞に存在するER (Estrogen Receptor) の過剰な活性化がTh2タイプのサイトカインの産生を促進し、Th1/Th2バランスがTh2にシフトするという内容である。３つ目は、「Inhibition of JAK3 leading to impairment of TDAR」というタイトルで、細胞内シグナル伝達に関与するJAK3の阻害により、IL-2およびIL-4の産生が抑制される結果、TDARが抑制されるという内容である。これらについてSPSF（Standard Project Submission Form）を作成し、2018年11月にOECDに提出した。その後に行われたEAGMSTの電話会議において、これらのAOP案が特に問題なく次のステップであるAOP wikiへの登録作業に進むこととなったため、原稿案を作成した。

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。また、これまで免疫毒性について成立したAOPはない。したがってこれまで作成したAOPをAOP wikiに登録するだけで終わりではなく、レビューアーのコメントに適切に対応しながら最終化していく必要がある。そのためには、OECDより発行されているUser’s Handbook supplement to the Guidance Document for developing and assessing Adverse Outcome Pathwaysなど関連情報を把握することはもちろん、EAGMSTの会議において直接情報交換を行うことが重要である。例えば、今年度新たに提案したER (Estrogen Receptor)に関するAOPでは、当初AOを「アレルギー応答の増悪」としていたが、EAGMASTの電話会議において「アレルギーはすでにAOであり、AOを増悪させるAOというのはわかりにくい」との指摘があったため、AOを「Th1/Th2バランスのTh2へのシフト」に修正した。

またJAK3阻害に関するAOPは、昨年度申請したカルシニューリン阻害のAOPと同じ「TDARの抑制」とした。これは、将来免疫毒性のAOPをネットワーク化する際、先行するAOPと関連があるAOPは審査の優先順位が高くなると判断したためである。

E. 結論

昨年度登録したAOPは内部レビューが終了し、外部レビューに移行することとなった。今年度新たに３つのAOPの開発をOECDに提案し、承認された。このように免疫毒性に関する様々なAOPを開発することで、将来の免疫毒性に関するAOPネットワーク構築に寄与していく。

F. 研究発表

F.1. 論文発表

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G. 知的財産権の出願・登録状況

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

平成30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

遺伝毒性の有害性発現経路（AOP: Adverse Outcome Pathway）開発にあたり、同 AOP への組み込みを想定した新規エピジェネティック毒性試験法「FLO assay」に関する研究を実施した。本年度は被検物質として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 を用いて、「FLO assay」の有用性を評価した。その結果、FLO assay は発がん性予測の精緻化に有効である可能性が示された。

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、研究分担者が独自開発に成功したエピジェネティック変異原検出系のプロトタイプ「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のエピジェネティック毒性評価を行い、発がん性AOPへのFLO assay活用の可能性を検証した。

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。使用した株の詳細はTable 1および2に示す。

B.2. 使用した化学物質

カビ毒オクラトキシンAとデオキシニバレノールは和光純薬工業（株）より購入した。デオキシニバレノールはIARCでグループ3（Not classifiable as to its carcinogenicity to humans）と分類される。

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。

B4. 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. Reverse-Transcription (RT)-PCR解析

SD -Trp/-Ura液体培地において対数増殖期中期から後期まで培養した酵母細胞よりRNAを抽出後、Super Script® One-Step RT-PCR with Platinum® Taq (Life technologies, USA)を使用しRT-PCRを行った。なお、使用したプライマーは以下の通りである。*FLO1*, 5′-CTCATCGCTATATGTTTTTGG-3′ (forward) および 5′-CGAGTAAACAACCTTCATTGG-3′ (reverse); *ACT1*, 5′-ATTCTGAGGTTGCTGCTTTGG-3′ (forward) および 5′-GAAGATTGAGCAGCGGTTTGC-3′ (reverse)。

B.6. *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.7. *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）に対するオクラトキシンA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.8. 核の蛍光染色

SD -Trp/-Ura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100 ng/mLの 4',6-diamidino-2-2-phenylindole (DAPI; 和光純薬工業（株）)で染色した。蛍光観察にはOlympus FluoView FV1000 with an IX81 inverted microscope（オリンパス（株））を使用した。

B.9. 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. オクラトキシン A 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に可逆的に応答する。そこでFLO assayとしてまずオクラトキシンA（12.5-50 μM）とデオキシニバレノール（50-200 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、オクラトキシンA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）の両株共に *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、pFIGSTpA（プロモーターとして DNA メチル化低感受性 *FLO1* プロモーターを有する）では認められなかった。一方、デオキシニバレノール処理では *FLO1* レポーター活性への影響は確認されなかった（Fig. 1）。

C.2. オクラトキシン A 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 として次にオクラトキシン A が凝集性に及ぼす影響を Empty-vector control strain と *DNMT* yeast の両株で検討した。その結果、凝集性を示す *DNMT* yeast においては、オクラトキシン A（12.5-50 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. 2）。

C.3. オクラトキシン A が *FLO1* mRNA レベルに及ぼす影響

Empty-vector control strain と *DNMT* yeast の両株を用いて、*FLO1* mRNA 転写レベルに対する OTA の作用を RT-PCR により確認した。その結果、オクラトキシン A（25-50 μM）の濃度依存的に *FLO1* mRNA レベルが減少することが明らかとなった（Fig. 3）。

C.4. DNA メチル化酵素に及ぼすオクラトキシン A 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。オクラトキシン A（50-200 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、オクラトキシン A の濃度上昇に伴い DNA メチル化活性の低下が認められることが確認された（Fig. 4）。

C.5. .酵母染色体構造に及ぼすオクラトキシン A の作用

オクラトキシン A が酵母核 DAPI 染色像に及ぼす影響を蛍光顕微鏡により観察した。その結果、*DNMT* yeast において特徴的な拡散した核の DAPI 染色像が、40 μM オクラトキシン A 処理により Empty-vector control strain の染色像に類似した状態に変化することが明らかとなった（Fig. 5）。

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とグループ3（Not classifiable as to its carcinogenicity to humans）のデオキシニバレノールの２種類のかび毒を被検物質として、それぞれの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。*FLO1* プロモーター誘導性レポーター活性に対してオクラトキシンAは濃度依存的に抑制したが、同効果はデオキシニバレノールでは認められなかった。また、オクラトキシンAによる本抑制作用はDNAメチル化低感受性*FLO1*プロモーター活性に対しては確認できなかった。*DNMT*yeastで顕在化する凝集性および *FLO1* mRNAレベルの上昇は共にオクラトキシンAにより抑制され、*in vitro*のアッセイではオクラトキシンA によりDNAメチル化酵素活性が阻害されることも明らかとなった。*DNMT*yeastで観察される異常な核染色像も、オクラトキシンA処理によりコントロール株に類似した核染色像に変化することが確認された。以上の結果は、オクラトキシンAがDNAメチル化阻害を毒性機序と

するエピジェネティック変異原であることを示唆している。

E. 結論

オクラトキシンAはIARCでグループ2B（Possibly carcinogenic to humans）と分類される。本研究において、オクラトキシンA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ヒトへの発がん性が危惧されるオクラトキシンA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、今回エピジェネティック変異原検出に使用したFLO assayが発がん性予測の精緻化に活用できる可能性も示している。

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F.1. 論文発表

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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	

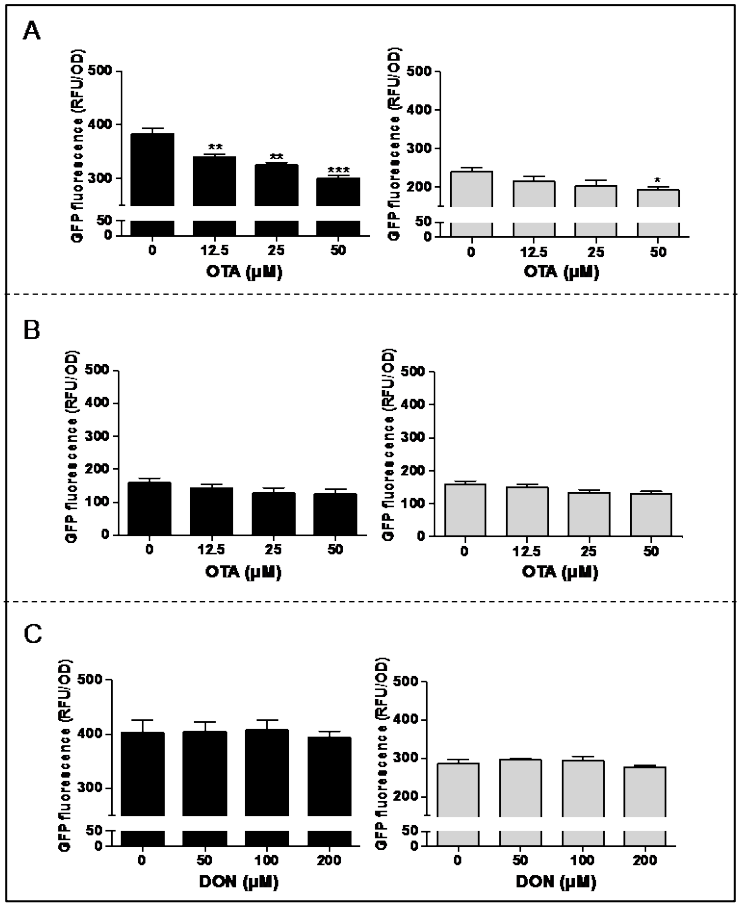

Fig. 1

FLO1 レポーター活性に対するオクラトキシン A（OTA）とデオキシニバレノール（DON）の効果 （A）pF1GS 形質転換 *DNMT* yeast（黒）または Empty-vector control strain（グ

レー）（B）pFIGSTpA 形質転換 *DNMT* yeast（黒）または Empty-vector control strain（グレー）（C）pF1GS 形質転換 *DNMT* yeast（黒）または Empty-vector control strain（グレー）

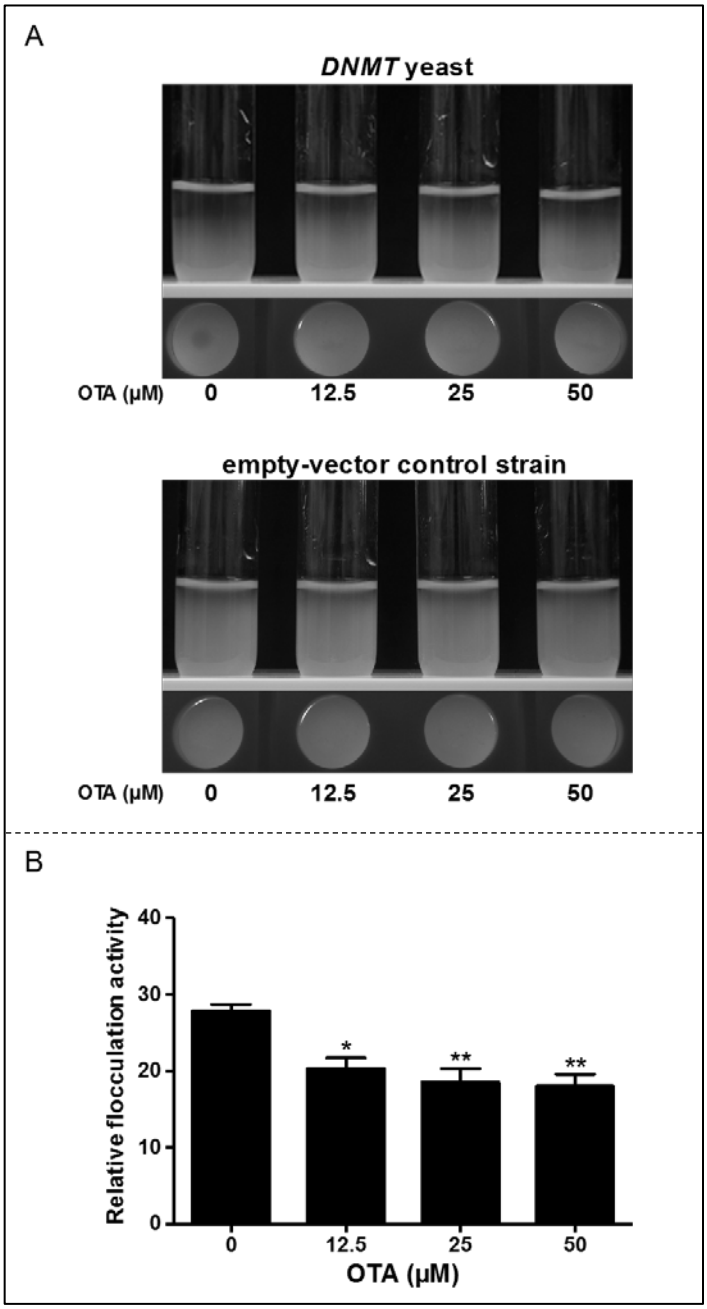

Fig. 2
凝集性に及ぼすオクラトキシン A（OTA）の作用 （A）凝集写真（B）*DNMT* yeast の相対的凝集活性

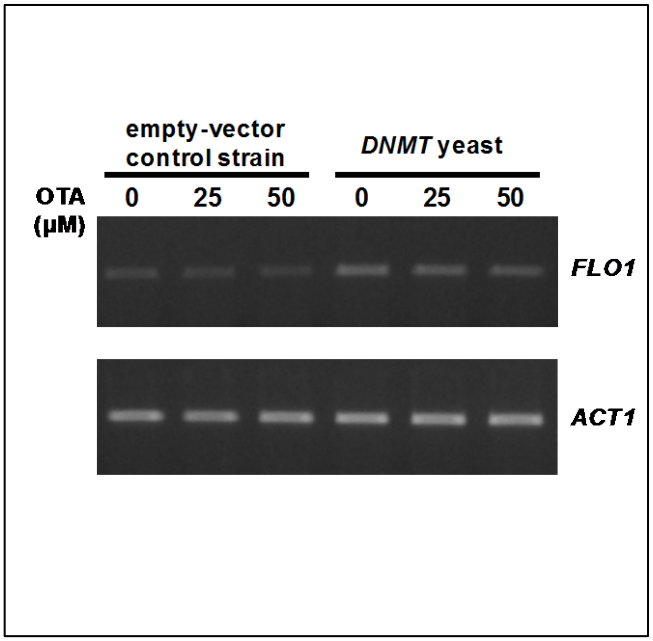

Fig. 3
FLO1 mRNA レベルに及ぼすオクラトキシン A（OTA）の作用

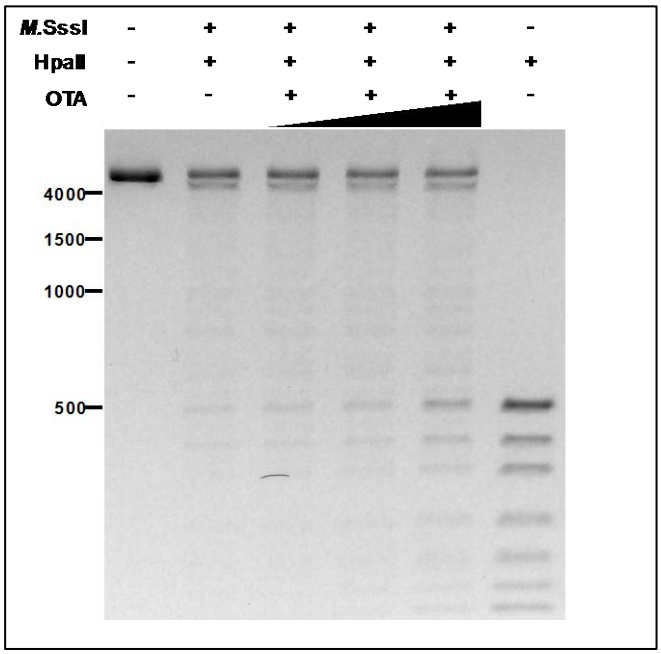

Fig. 4
オクラトキシン A（OTA）が *M*.SssI 活性に及ぼす影響　左端の数字は DNA のサイズ（bp）

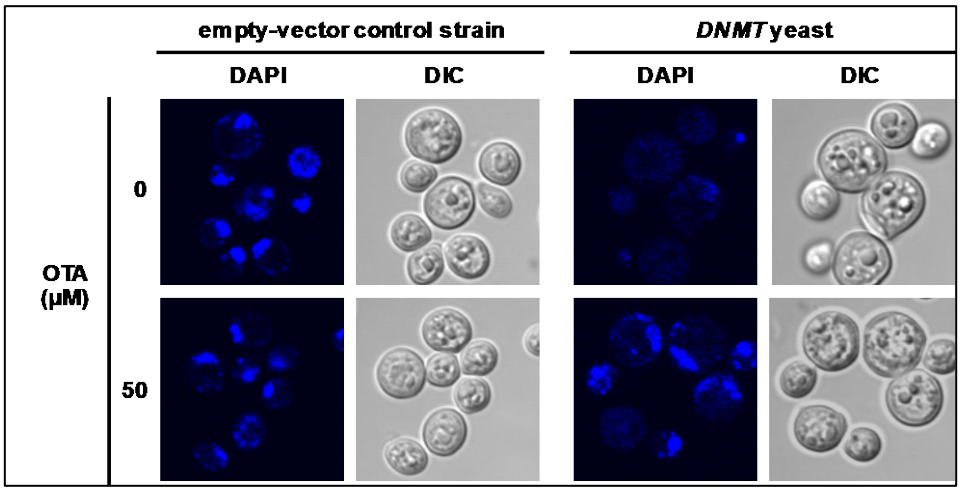

Fig. 5

オクラトキシン A（OTA）が酵母核染色像に及ぼす影響

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

平成30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。代償性反応の重要性は Adverse Outcome Pathway（AOP）開発プログラムにおいても説かれており、代償性反応に関する情報は Key event 間の定量的な理解に有用であるとされている。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代償性肥大が知られている。この代償性肥大は腎障害の発現機序に関わらず共通して生じる現象であることから、代償性肥大の発現機序を詳細に理解することにより、新たな腎毒性評価分子を抽出することも期待できる。本研究では 10 週齢の雌雄 F344 ラットに片側腎摘出を施し、残存腎における代償性肥大の分子機構を解明し、腎毒性の AOP 作成に資する情報を得るとともに、新しい腎障害評価分子を抽出することを目的とした。

病理組織学的および遺伝子発現解析の結果、雌雄ともに残存腎における細胞増殖活性が対照群と比して上昇しており、細胞周期停止に関わる transforming growth factor（TGF）-β1 の mRNA 発現に処置による影響はみられなかった。mRNA 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雄では 320 個、雌では 233 個の遺伝子の発現が変動していた。パスウェイ解析の結果、雌雄ともに主に細胞増殖に関連するパスウェイの活性化が認められた。microRNA（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。

以上の結果から、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明らかとなった。また miRNA マイクロアレイの結果から、腎臓の代償性機構における細胞増殖活性は miRNA に制御されていることが示唆され、これらの因子は新しい腎障害評価分子になることが期待された。今後、mRNA-miRNA 統合解析を実施し、腎代償性機構における miRNA の細胞周期制御機構について詳細に検討する予定である。

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反応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非常に重要である。

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機構の理解に対する重要性が説かれており、代償性反応に関する情報はKey Event間の関係の定量的な理解に有用であるため、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、その原因としては腎血流量が豊富であるために化学物質に曝されやすいこと、尿の精製過程において原尿中の化学物質が濃縮されること、さらに尿細管内には多くの薬物代謝酵素が存在することなどが挙げられる。腎臓は多様な細胞により構成される臓器であるが、なかでも尿細管は尿細管腔から原尿中の化学物質を再吸収・代謝するという生物学的特徴から、最も障害を受けやすい細胞である。

腎臓には大きく2つの代償性機構が内在する。腎毒性物質による障害はネフロン単位で生じ、障害を受けたネフロンにおける代償性反応としては再生尿細管による組織の修復が知られている。一方、非障害ネフロンではいわゆる代償性肥大が生じる。腎毒性発現機序には種々の様式があることに対し、この代償性肥大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、代償性肥大の発現機序を詳細に理解することにより、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。

片側腎摘出モデル動物は腎代償性肥大の機序解明に汎用されているモデルであるが、その分子機構について一定の見解は得られていない。この理由としては、これまでの研究では種々の週齢および性別のラットおよびマウスを用いていることが考えられる。よって、安全性評価に資する情報を得るためには、毒性試験に汎用される週齢の雌雄ラットを用いた機序検討が有用であると考えた。

本研究では10週齢の雌雄F344ラットに片側腎摘出を施し、残存腎における代償性肥大の分子機構を解明し、腎毒性のAOP作成に資する情報を得るとともに、新しい腎障害評価分子を抽出することを目的とした。

平成30年度は雌雄ラットに片側腎摘出術を施して残存腎組織を採材し、細胞増殖活性を免疫組織学的および遺伝子発現解析により検索し、さらに網羅的遺伝子発現解析によりmRNAおよびmicroRNA（miRNA）の発現を解析した。

B. 研究方法

10週齢の雌雄F344ラットをそれぞれ4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および3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 stain（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、real time RT-PCR

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処置後2日および対照群のサンプルを用いて実施した。さらにIngenuity Pathways Analysis（IPA）により変動のあったmRNAについてパスウェイ解析を実施した。

腎臓の代償性機構の雌雄差をさらに検討するため、15週齢の雌雄F344ラットを3群（n=5）に配し、同様に片側腎摘出術を施して処置後2および3日の右腎臓においてBrdU陽性細胞率の算出およびreal time RT-PCRを実施した。

（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

雌雄ともに片側腎摘出後の残存腎の重量が処置後2および3日に対照群と比して有意な増加を示した。残存腎の組織学的解析では、処置後2および3日において近位曲尿細管、近位直尿細管および遠位尿細管のBrdU陽性細胞率が対照群と比して有意な上昇あるいは上昇傾向を示した。mRNA発現解析においてはcyclin E1の発現が雌雄ともに処置後1から3日で有意な上昇あるいは上昇傾向を示し、処置後2日が最も高値を示した。また、雌雄ともにtransforming growth factor（TGF）-β1のmRNA発現に処置による影響はみられなかった。mRNA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雄では320個、雌では233個の遺伝子の発現が変動していた。IPAを用いたNew Comparison Analysisの結果、雌雄ともに主に細胞増殖に関わるCanonical Pathwayの活性化が認められた。

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（miR-1843a-5p，miR-1843a-3p，miR-194-3p，miR-222-3p，miR-31-5p，miR-340-5p, miR-450-5p, miR-653-5p, miR-9a-3p）発現が変動しており、全て発現低下を示した。

15週齢の雌雄F344ラットを用いた実験においても同様に、雌雄ともに対照群と比して処置後2および3日後の腎臓において重量の有意な増加、BrdU陽性細胞率の有意な増加およびCyclin E1の遺伝子発現の有意な上昇が認められた。一方、TGF-β1の遺伝子発現に処置による影響はみられなかった。

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）が寄与しているとされている。細胞周期のG1/S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、G1/S期移行に関与するCyclin E1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ことから、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。

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。miRNAはmRNAの3'-UTRへの結合を介してmRNAを分解することにより、mRNAの機能を転写後に抑制する。本実験結果では多数の細胞増殖関連遺伝子のmRNA発現上昇がみられたことから、発現が低下していたmiRNAは細胞周期を上流で制御している可能性が示唆された。

性成熟のさらに進んだ15週齢の雌雄ラットを用いて検討した結果、同様にBrdU陽性細胞率およびCyclin E1の遺伝子発現の上昇がみられ、TGF-β1の遺伝子発現変動はみられなかったことから、少なくとも本実験条件下では腎臓の代償性機構に雌雄差はないと考えられた。

E. 結論

雌雄ラットの腎代償性機構には細胞の増数（過形成）が寄与していることを明らかにした。また細胞増殖活性の亢進はmiRNAにより制御されていることが示唆され、これらの因子は新しい腎障害評価分子になる可能性が期待された。今後、mRNA-miRNA統合解析を実施し、腎代償性機構におけるmiRNAの細胞周期制御機構について詳細に検討する予定である。

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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

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

平成30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）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、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。そこで本研究では、平成 29 年度に終了し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 3 課題で得られた基礎試験データを、公的データベースおよび *in silico* ツールから抽出した化学物質情報、*in silico・in vitro* 分子プロファイリング情報、毒性アラート情報、毒性試験情報、法関連情報とともに集積・統合してデータベース化するために、データベースのデータ項目と収集するデータソースを設定した。そして 1 課題を選択して一部の物質についてデータ入力を試行した。次年度以降は、当該課題について、全データ入力を完了させるとともに、残りの 2 課題の化学物質リスク研究事業で試験された物質を対象にデータ集積を行い、データベース化を行う。毒性情報収集の効率化のため、OECD の eChemPortal などの情報源の利用も検討するとともに、データ項目の追加・再検討を行う。

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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。

国際的には、IATA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、日常生活に利用される種々の化学物質のリスク評価、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。昨今は、情報源としての種々の化学、バイオ、毒性データベースや*in silico*ツールなどの開発が進むが、データが散在しており、また日常利用していないユーザーにとってはツールの使い方が簡単でない場合もあることから、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は難しい。

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、化学物質情報、*in silico*・*in vitro*分子プロファイリング情報、毒性試験情報、法関連情報とともに集積・統合したデータベースを作成してデータを集積し、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

以下の厚生労働科学研究化学リスク研究事業総合報告書情報を入手して調査し、データ収集と解析の対象化学物質と実施された試験をリスト化した(http://mhlw-grants.niph.go.jp/niph/search/NIFL00.do)。

1）免疫毒性評価試験法Multi-Immuno Toxicity assayの国際validationへ向けての検討 [H27-化学-一般-006]（研究代表者 相場節也）
2）発生-発達期における低用量の化学物質曝露による成熟後の神経行動毒性の誘発メカニズム解明とその毒性学的評価系構築に資する研究 [H27-化学-一般-007]（研究代表者 種村健太郎）
3）新規*in vitro*評価系とマーカーの開発によるナノマテリアルのリスク評価及びリスク低減化に関する研究 [H27-化学-

一般-008]（研究代表者 渡邉昌俊）

B.2. 化学物質リスク研究事業試験研究対象物質の関連情報の収集

上記化学物質リスク研究事業試験研究対象物質の物質情報、化学反応性、生物学的反応性のプロファイリング、法施行関連情報を収集するために、以下の公共データベースと*in silico*ツールを使用した。

１）ChemSpider (Royal Society of Chemistry) (http://www.chemspider.com/)
２）PubChem (National Center for Biotechnology Information) (https://pubchem.ncbi.nlm.nih.gov/)
３）NITE 化学物質総合情報提供システム (CHRIP; https://www.nite.go.jp/chem/chrip/chrip_search/systemTop)
４）OECD QSAR Toolobox (http://www.oecd.org/chemicalsafety/risk-assessment/oecd-qsar-toolbox.htm)

C. 研究結果

C-1. データ収集と解析対象の化学物質

免疫毒性試験法開発、神経毒性評価系構築、ナノマテリアル *in vitro* 評価系構築の 3 研究事業について、データ収集・解析対象物質とそれらが供せられた試験・解析系を整理した（表 1 (A)~(C)）。そのうち、免疫毒性評価試験法 Multi-Immuno Toxicity assay (MITA)の開発研究は、試験プロトコールが確立され、多くの物質が並列で試験されてデータ集積が進んでいる。物質間でプロファイルや試験データを体系的に相互比較しやすいと考えられたことから、本研究事業報告書を取り上げて、毒性関連情報を集約したデータベース構築のためのデータ項目とデータソースを検討した。

C-2. データベースのデータ項目の設定

収集したデータは、Excel (Microsoft 社)及び化学物質関連情報を収載するデータベースソフトウェアとして広く利用されている ChemFinder (Hulinks 社)、InstantJChem (ChemAxon 社)で閲覧・検索することを想定し、Excel 形式で入力した。データ項目として、以下を仮設定した。

１）化学物質情報（CAS No., 物質名称（日本語名、英語名）、SMILES（ChemSpider, PubChem 形式）、InChI, InChI Key）
２）物理化学情報（分子量、logP）
３）化学反応性情報（OECD QSAR Toolbox による生体分子との反応性に関するプロファイリングデータ）
４）毒性アラート情報（OECD QSAR Toolbox ならびに DEREK Nexus による遺伝毒性、発がん性、皮膚感作性アラート）
５）生物学的反応性情報（免疫毒性評価試験 Multi-ImmunoTox assay; MITA 、ToxCast、Tox21 など *in vitro* バイオアッセイ試験結果）
６）毒性情報（遺伝毒性、反復投与毒性、生殖発生毒性、発がん性、免疫毒性）
７）法施行関連情報（化審法、化管法、安衛法、毒劇法、大防法、REACH SVHC、TSCA、国内外有害性評価書またはリスク評価書情報）

これまでに 20 物質のデータを試行的に入力した。CAS 95-80-7 の例を表 2 に示す。

D. 考察

本分担研究では、厚生労働科学研究化学物質リスク研究事業で実施された成果の基礎試験データを集積することにより、今後の効率的な試験戦略の策定や規制行政に役立てるため、*in silico*、*in vitro*、*in vivo*のデータを集約・統合したデータベースの基本構造を検討した。

図2に、本データベースの利用シナリオの例を示す。厚生労働科学研究化学物質リスク研究事業で評価対象となる化学物質に関連して、モニタリング情報、事故情報、海外規制動向等に基づき、評価・管理する化学物質のインベントリーから安全性評価を実施する必要がある化学物質が設定されたとき、評価対象物質ならびにその関連物質について、*in silico*ツールを用いた分子プロファイリング、毒性アラート情報と当該データベースの基礎試験データ、種々の*in vitro*バイオアッセイ試験データ、 *in vitro*/*in vivo*毒性試験情報を収集し、これらをWoEにより統合的に解析し、評価・審議支援、試験戦略を策定することにより規制上の結論を得る。既存の情報が不十分な場合は、効率的に必要な試験データを取得したうえで、規制上の決定を下す。例えば表2は、2,4-diaminotolueneは、*in silico*、*in vitro*分子プロファイリングにより生体分子との反応性が高く、また警告構造を有していることを示している。免疫毒性の他、種々の毒性試験結果より、ヒト健康影響が懸念され、国内外で種々の法規制が行われている。Diaminobenzene誘導体が評価対象物質となった時には、免疫毒性、遺伝毒性、発がん性の観点から、*in silico*プロファイリングと毒性アラートの抽出を行い、その予測結果の不確実性が大きいと考えられる場合には、必要な*in vitro*試験を実施してデータを収集し、さらにより精緻な評価が必要な時にはデータが欠損する*in vivo*毒性を実施するといったように、段階に応じた評価と規制上の判断へ利用することが考えられる。次年度以降は、神経毒性評価のために試験された物質を対象にデータベース化を行う。毒性情報収集の効率化のため、OECDのeChemPortalなどの利用も検討する。

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プラットフォームである。新たに開発する毒性予測モデルに加えて、安全性予測判断に有用な文献等のテキスト情報検索機能の実装を計画している。将来的には、AI-CSAFEも情報収集源として追加することを検討する。

E. 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・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開発に役立てるため、その基礎試験データを、化学物質情報、*in silico*・*in vitro*分子プロファイリング情報、毒性アラート、毒性試験情報、法関連情報とともにデータベース化するためのデータ項目およびデータソースを設定した。

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G. 知的財産権の出願・登録状況

なし

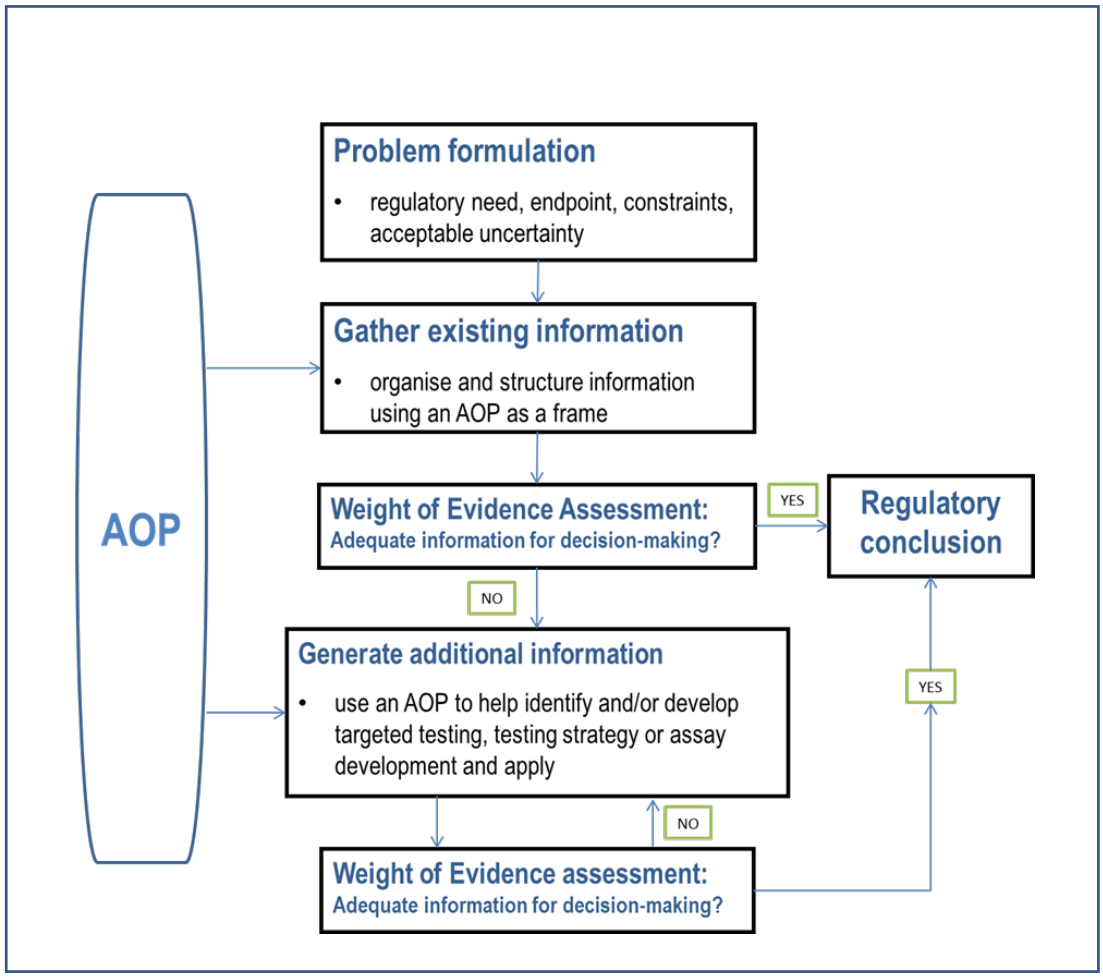

図1　OECDが提唱する規制判断のためのIATAワークフロー

表1　3研究課題でデータ収集と解析対象となった化学物質のリスト

（A）研究課題名：平成 27～29 年度　免疫毒性評価試験法 Multi-Immuno Tox assay の国際 validation へ向けての検討

No	物質名	No	物質名
1	2,4-Diaminotoluene	31	Mercuric chloride
2	2-Aminoanthracene	32	Methanol
3	2-Mercaptobenzothiazole	33	Mitomycin C
4	Acetaminophen	34	Nickel sulfate
5	Actinomycin D	35	Nitrofurazone
6	Aluminum chloride	36	Pentamidine isethionate
7	Amphotericin B	37	p-Nitroaniline
8	Benzethonium chloride	38	Pyrimethamine
9	Chlorpromazine	39	Ribavirin
10	Cisplatin	40	Sodium bromate
11	Cobalt chloride	41	Sodium dodecyl sulfate
12	Cyclophosphamide	42	Triethanolamine
13	Cyclosporine A	43	Hexachlorobenzene
14	Dapsone	44	Citral
15	Dexamethasone	45	Trichloroethylene
16	Dibenzopyrene	46	Chloroplatinic acid
17	Dibutyl phthalate	47	Formaldehyde
18	Diethanolamin	48	Diesel exhaust particles
19	Dimethyl sulfoxide	49	Azathioprine
20	Ethanol	50	Chloroquine
21	FK 506	51	Colchicine
22	FR167653	52	Digoxin
23	Histamine	53	Methotrexate
24	Hydrocortisone	54	Minocycline
25	Hydrogen peroxide	55	Mizoribine
26	Isoniazid	56	Mycophenolic acid
27	Isophorone diisocyanate	57	Nicotinamide
28	Lead(II) acetate	58	Rapamycin
29	Lithium carbonate	59	Sulfasalazine
30	Magnesium sulfate	60	Warfarin

（B）研究課題名：平成 27～29 年度　発生-発達期における低用量の化学物質暴露による成熟後の神経行動毒性の誘発メカニズム解明とその毒性学的評価系構築に資する研究

No	物質名	分担研究課題名
1	アセタミプリド	周産期における低用量ネオニコチノイド系農薬暴露による成熟後マウスの行動異常、およびエストロジェン受容体遺伝子改変マウスを用いた行動異常値設定の試み
		行動異常標準マウス脳の遺伝子発現解析
		神経幹細胞動態解析および大脳層構造形態解析と異常基準値の設定、新規毒性マーカー探索
		光計測による神経回路解析の神経毒性評価への応用
2	イミダクロプリド	周産期における低用量ネオニコチノイド系農薬暴露による成熟後マウスの行動異常、およびエストロジェン受容体遺伝子改変マウスを用いた行動異常値設定の試み
		行動異常標準マウス脳の遺伝子発現解析
		神経幹細胞動態解析および大脳層構造形態解析と異常基準値の設定、新規毒性マーカー探索
		光計測による神経回路解析の神経毒性評価への応用
3	無機ヒ素	行動柔軟性/抑制課題試験の開発と毒性評価への応用
4	ドーモイ酸	周産期における低用量ネオニコチノイド系農薬暴露による成熟後マウスの行動異常、およびエストロジェン受容体遺伝子改変マウスを用いた行動異常値設定の試み
5	イボテン酸	周産期における低用量ネオニコチノイド系農薬暴露による成熟後マウスの行動異常、およびエストロジェン受容体遺伝子改変マウスを用いた行動異常値設定の試み
6	アセフェート	周産期における低用量ネオニコチノイド系農薬暴露による成熟後マウスの行動異常、およびエストロジェン受容体遺伝子改変マウスを用いた行動異常値設定の試み
7	バルプロ酸	神経幹細胞動態解析および大脳層構造形態解析と異常基準値の設定、新規毒性マーカー探索
		光計測による神経回路解析の神経毒性評価への応用
8	ビスフェノールA	光計測による神経回路解析の神経毒性評価への応用

（C）研究課題名：平成 27～29 年度　新規 *in vitro* 評価系とマーカーの開発によるナノマテリアルのリスク評価およびリスク低減化に関する研究

No	物質名	分担研究課題名
1	非修飾磁性体ナノ粒子 (Fe3O4 NPs)	切片担体培養系を用いたナノマテリアルのリスク評価系の構築・エピジェネティクスマーカーの検索・ナノマテリアルの細胞内動態の解析
		3D皮膚モデルを用いたナノマテリアルの経皮毒性評価系構築
		ナノマテリアル曝露における網羅的遺伝子発現解析
2	磁性体ナノ粒子 (Fe3O4 NPs-COOH)	切片担体培養系を用いたナノマテリアルのリスク評価系の構築・エピジェネティクスマーカーの検索・ナノマテリアルの細胞内動態の解析
		3D皮膚モデルを用いたナノマテリアルの経皮毒性評価系構築
		ナノマテリアル曝露における網羅的遺伝子発現解析
3	金ナノ粒子	ナノマテリアルの作製およびキャラクタリゼーション
		3D皮膚モデルを用いたナノマテリアルの経皮毒性評価系構築
4	銀ナノ粒子	ナノマテリアルの作製およびキャラクタリゼーション
		3D皮膚モデルを用いたナノマテリアルの経皮毒性評価系構築
5	酸化チタンナノ粒子 (TiO2 NPs)	ナノマテリアルの作製およびキャラクタリゼーション
6	MWCNT-S	共培養系及び 3D 皮膚モデルを用いたナノマテリアルの遺伝毒性評価系の構築
		ナノマテリアル曝露における網羅的遺伝子発現解析
7	MWCNT-L	共培養系及び 3D 皮膚モデルを用いたナノマテリアルの遺伝毒性評価系の構築
		ナノマテリアル曝露における網羅的遺伝子発現解析
8	MGT(BMS-10)	共培養系及び 3D 皮膚モデルを用いたナノマテリアルの遺伝毒性評価系の構築
9	MGT(BMSC-5)	共培養系及び 3D 皮膚モデルを用いたナノマテリアルの遺伝毒性評価系の構築
10	フォルペット（N-（トリクロロメチルチオ）フタルイミド）	3D皮膚モデルを用いたナノマテリアルの経皮毒性評価系構築
11	酸化亜鉛（ZnO）ナノマテリアル	ナノマテリアルの細胞毒性及び遺伝毒性発現メカニズムの解析
12	酸化ニッケル（NiO)ナノマテリアル	ナノマテリアルの細胞毒性及び遺伝毒性発現メカニズムの解析
		細胞応答に及ぼすナノマテリアルの物性解析
13	Niナノマテリアル	細胞応答に及ぼすナノマテリアルの物性解析
14	Niイオン	細胞応答に及ぼすナノマテリアルの物性解析

表２　データベースのデータ項目とデータ入力例

カテゴリー	データ項目	データソース	対象物質
物質情報	CAS	ChemSpider	95-80-7
	物質名称	報告書	2,4-Diaminotoluene
	物質名称（NITE CHRIP日本語）	NITE CHRIP	４－メチル－１，３－フェニレンジアミン
	物質名称（ACD/IUPAC name）	ChemSpider	4-Methyl-1,3-benzenediamine
	SMILES	ChemDraw	NC1=CC(N)=CC=C1C
	SMILES	PubChem	CC1=C(C=C(C=C1)N)N
	InChI	ChemSpider	InChI=1S/C7H10N2/c1-5-2-3-6(8)4-7(5)9/h2-4H,8-9H2,1H3
	InChI Key	ChemSpider	VOZKAJLKRJDJLL-UHFFFAOYSA-N
物理化学情報	MW	PubChem	122.171 g/mol
	XLogP	PubChem	0.1
	LogP	PubChem	0.14, 0.35
化学反応性	DNA binding by OASIS	QSAR Toolbox	Radical|Radical >> Radical mechanism via ROS formation (indirect)|Radical >> Radical mechanism via ROS formation (indirect) >> Single-Ring Substituted Primary Aromatic Amines|SN1|SN1 >> Nucleophilic attack after nitrenium ion formation|SN1 >> Nucleophilic attack after nitrenium ion formation >> Single-Ring Substituted Primary Aromatic Amines
	DNA binding by OECD	QSAR Toolbox	SN1|SN1 >> Nitrenium Ion formation|SN1 >> Nitrenium Ion formation >> Primary aromatic amine
	Protein binding by OASIS	QSAR Toolbox	AN2|AN2 >> Michael-type addition to quinoid structures |AN2 >> Michael-type addition to quinoid structures >> Substituted Anilines
	Protein binding by OECD	QSAR Toolbox	No alert found
	Protein binding potency	QSAR Toolbox	Not possible to classify according to these rules (GSH)
	Protein binding potency Cys	QSAR Toolbox	-
	Protein binding potency Lys	QSAR Toolbox	-
毒性アラート	DNA alerts for AMES by OASIS	QSAR Toolbox	Radical|Radical >> Radical mechanism via ROS formation (indirect)|Radical >> Radical mechanism via ROS formation (indirect) >> Single-Ring Substituted Primary Aromatic Amines|SN1|SN1 >> Nucleophilic attack after nitrenium ion formation|SN1 >> Nucleophilic attack after nitrenium ion formation >> Single-Ring Substituted Primary Aromatic Amines
	DNA alerts for CA and MNT by OASIS	QSAR Toolbox	Radical|Radical >> Radical mechanism via ROS formation (indirect)|Radical >> Radical mechanism via ROS formation (indirect) >> Single-Ring Substituted Primary Aromatic Amines|SN1|SN1 >> Nucleophilic attack after nitrenium ion formation|SN1 >> Nucleophilic attack after nitrenium ion formation >> Single-Ring Substituted Primary Aromatic Amines
	Keratinocyte gene expression	QSAR Toolbox	Not possible to classify according to these rules
	Protein binding alerts for Chromosome aberration by OASIS	QSAR Toolbox	AN2|AN2 >> Michael addition to the quinoid type structures|AN2 >> Michael addition to the quinoid type structures >> Substituted Anilines
	Protein binding alerts for skin sensitization according to GHS	QSAR Toolbox	-
	Protein binding alerts for skin sensitization by OASIS	QSAR Toolbox	No alert found
	Protein binding potency h-CLAT	QSAR Toolbox	
	Respiratory sensitization	QSAR Toolbox	No alert found

カテゴリー	データ項目	データソース		対象物質
	DEREK ames mutagenicity	DEREK Nexus		plausible (Aromatic amine or amide)
	DEREK skin sensitization	DEREK Nexus		plausible (Aromatic primary or secondary amine)
	DEREK carcinogenicity	DEREK Nexus		plausible (Aromatic amine or amide)
物理化学実測	Chemical reactivity COLIPA	QSAR Toolbox		-
	GSH Experimental RC50	QSAR Toolbox		-
生物学的反応性 (in vitro)	MITA	IL-2		Judge : No effect LOEL : -
	MITA	IFN-γ		Judge : immunoaugmentation LOEL : 62.5
	MITA	IL-1β		Judge : No effect LOEL : -
	MITA	IL-8		Judge : immunosuppression LOEL : 0.98
	MITA	IL-8 Luc		Judge : No effect LOEL : -
	PREPL - prolyl endopeptidase like (human)	The Scripps Research Institute Molecular Screening Center	2751	Active
	AHR - aryl hydrocarbon receptor (human)	NCGC	651777	Active
	AHR - aryl hydrocarbon receptor (human)	Tox21	743085	Active
	AHR - aryl hydrocarbon receptor (human)	Tox21	743085	Active
	AHR - aryl hydrocarbon receptor (human)	Tox21	743122	Active
	AHR - aryl hydrocarbon receptor (human)	Tox21	743122	Active
	RARA - retinoic acid receptor alpha (human)	Tox21	1159553	Active
	NR1I3 - nuclear receptor subfamily 1 group I member 3 (human)	Tox21	1224839	Active
	NR1I3 - nuclear receptor subfamily 1 group I member 3 (human)	Tox21	1224892	Active
	NR1I3 - nuclear receptor subfamily 1 group I member 3 (human)	Tox21	1224892	Active
	ESRRA - estrogen related receptor alpha (human)	Tox21	1224849	Active
	ESRRA - estrogen related receptor alpha (human)	Tox21	1259402	Active
	ESRRA - estrogen related receptor alpha (human)	Tox21	1259404	Active
	ESRRA - estrogen related receptor alpha (human)	Tox21	1224849	Active
	ESRRA - estrogen related receptor alpha (human)	Tox21	1259402	Active
	ESRRA - estrogen related receptor alpha (human)	Tox21	1259404	Active
	ESRRA - estrogen related receptor alpha (human)	Tox21	1224849	Active
	ESRRA - estrogen related receptor alpha (human)	Tox21	1259402	Active
	ESRRA - estrogen related receptor alpha (human)	Tox21	1259404	Active
毒性情報	Ames	JECDB		-
	CA	JECDB		-
	MN	JECDB		-
	RDT	HESS		-
	DART	NIHS		-

カテゴリー	データ項目	データソース	対象物質
	carcinogeincity	QSAR Toolbox	positive
	Bacterial mutagenicity	QSAR Toolbox	positive
	chromosome aberration	QSAR Toolbox	positive
	skin sensitization	QSAR Toolbox	weak
	Immunotoxicity	HSDB	suppression
法施行関連情報	化学物質の審査及び製造等の規制に関する法律（化審法）	NITE CHRIP	難分解性
	化学物質の審査及び製造等の規制に関する法律（化審法）		低濃縮性
	特定化学物質の環境への排出量の把握等及び管理の改善の促進に関する法律（化管法）		第一種
	労働安全衛生法（安衛法）		安衛法：強い変異原性が認められた化学物質
	毒物及び劇物取締法		政令・劇物
	大気汚染防止法		有害大気汚染物質
	国連番号（UN Number）／国連危険物分類 （UN Hazard Class）		6.1
	REACH：高懸念物質（SVHC）		Candidate List
	米国：有害物質規制法（TSCA）		ACTIVE
	中国：危険化学品目録（２０１５版）		２，４－ジアミノトルエン
	韓国：化評法（K-REACH）／化管法：有害化学物質、重点管理物質		有毒物質
	政府によるGHS分類結果		2,4-トルエンジアミン (別名: 2,4-ジアミノトルエン)
	化学物質有害性評価書／初期リスク評価書		https://www.nite.go.jp/chem/chrip/dt/pdf/CI_02_001/risk/pdf_gaiyou/228gaiyou.pdf
	環境省化学物質の環境リスク評価結果		http://www.env.go.jp/chemi/report/h16-01/pdf/chap02/02_2_29.pdf
	安衛法：リスク評価実施物質		https://www.mhlw.go.jp/shingi/2009/06/dl/s0630-7at.pdf
	OECD：高生産量化学物質（HPV Chemicals）		https://hpvchemicals.oecd.org/ui/search.aspx
	国際化学物質安全性カード（ICSC）		http://www.ilo.org/dyn/icsc/showcard.display?p_lang=en&p_card_id=0582&p_version=2
	環境保健クライテリア（EHC）		http://www.inchem.org/documents/ehc/ehc/ehc74.htm
	米国環境保護庁（EPA）：統合リスク情報システム（IRIS）		https://cfpub.epa.gov/ncea/iris2/chemicalLanding.cfm?&substance_nmbr=536
	EU：リスク評価書（RAR）		https://echa.europa.eu/web/guest/information-on-chemicals/information-from-existing-substances-regulation
	ドイツ化学会諮問委員会（BUA）：リスク評価書		https://www.gdch.de/publikationen/weitere-publikationen.html
	日本産業衛生学会：発がん分類		2B
	国際がん研究機関（IARC）：発がん性評価		2B
	米国国家毒性計画（NTP）：発がん性評価		R
	EU：発がん性評価		1B
	微生物を用いた変異原性試験（エームズ試験）結果		陽性
	CHL/IU細胞を用いた染色体異常試験結果		陽性

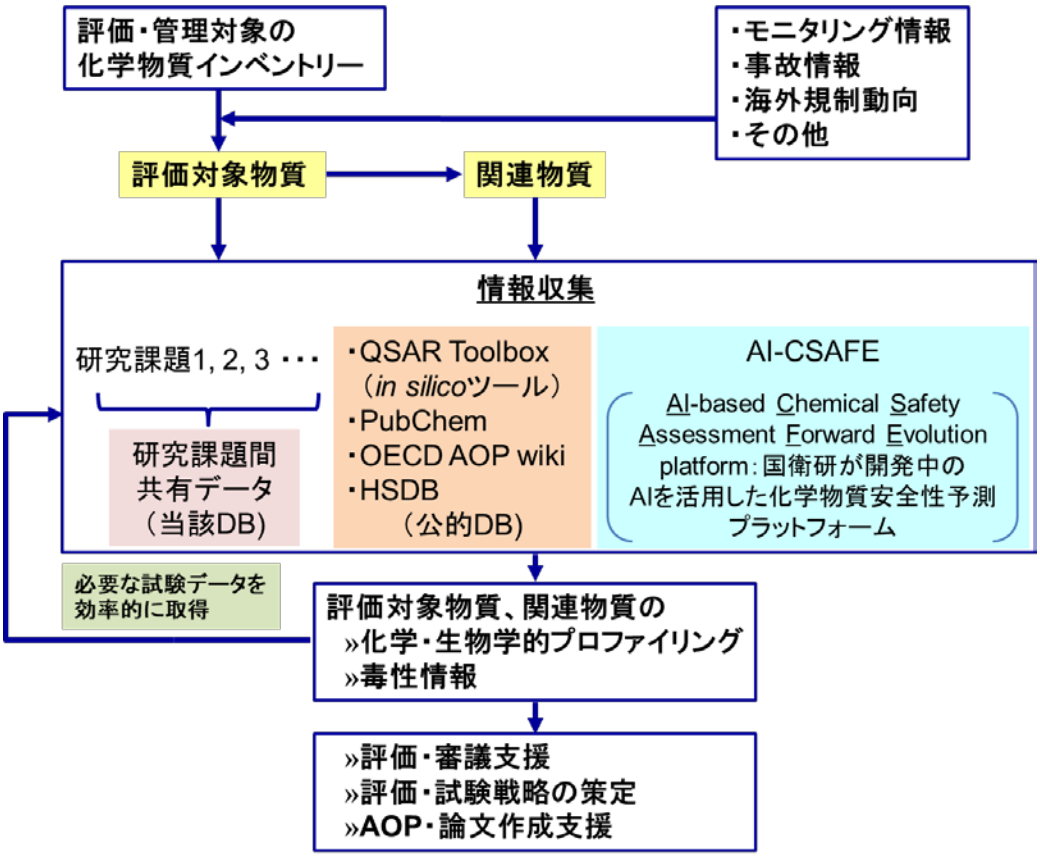

図２　本データベースの想定される利用シナリオの例

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
小島　肇	皮膚・粘膜毒性	日本トキシコロジー学会教育委員会	トキシコロジー第３版	朝倉出版	東京	2018	279-286
小島　肇	動物実験代替法	日本トキシコロジー学会教育委員会	トキシコロジー第３版	朝倉出版	東京	2018	320-325
小島　肇	in vitro実験の重要性と培養細胞の選択方法	古江美保（楠田），関野祐子	創薬のための細胞利用技術の最新動向と市場	シーエムシー・リサーチ	東京	2018	3-6
山田隆志	OECDにおけるQSAR、AOPの開発状況		皮膚の安全性・有用性評価法	技術情報協会	東京	2018	151-157

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
Fujita M, Yamamoto Y, Watanabe S, Sugawara T, Wakabayashi K, Tahara Y, Horie N, Fujimoto K, Kusakari K, Kurokawa Y, Kawakami T, Kojima K, Kojima H, Ono A, Katsuoka Y, Tanabe H, Yokoyama H, Kasahara T	Cause of and countermeasures for oxidation of the cysteine-derived reagent used in the amino acid derivative reactivity assay	J Appl Toxicol	39(2)	191-208	2019
Mitachi T, Kouzui M, Maruyama R, Yamashita K, Ogata S, Kojima H, Itagaki H	Some non-sensitizers upregulate CD54 expression by activation of the NLRP3 inflammasome in THP-1 cells	J Toxicol Sci.	44(3)	213-224	2019
小島　肇	化学物質の毒性評価方法の現状と今後，化学物質と環境	エコケミストリー研究会	154	1-3	2019

Sone M, Toyoda T, Cho YM, Akagi JI, Matsushita K, Mizuta Y, Morikawa T, Nishikawa A, Ogawa K	Immunohistochemistry of γ-H2AX as a method of early detection of urinary bladder carcinogenicity in mice	J. Appl. Toxicol.	in press		2019
Toyoda T, Matsushita K, Morikawa T, Yamada T, Miyoshi N, Ogawa K	Distinct differences in the mechanisms of mucosal damage and γ-H2AX formation in the rat urinary bladder treated with *o*-toluidine and *o*-anisidine	Arch Toxicol	93	753-762,	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	1303-1308	2019
Sugiyama KI, Furusawa H, Grúz P, Kinoshita M, Honma M.	Inhibitory effect of ochratoxin A on DNMT-mediated flocculation of yeast.	Mutagenesis	in printing	in printing	2019
Petkov PI, Schultz TW, Honma M. Yamada T, Kaloyanova E, Mekenya OG.	Validation of the performance of TIMES genotoxicity models with EFSA pesticide data.	Mutagenesis	34	83-90	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.	OECD	*in press*		2019
中村和昭, 諫田泰成, 山崎大樹, 片岡 健, 青井貴之, 中川誠人, 藤井万紀子, 阿久津英憲, 末盛博文, 浅香 勲, 中村幸夫, 小島 肇, 伊藤弓弦, 関野祐子, 古江－楠田美保	「培養細胞の観察の基本原則」の提案	組織培養研究	37(2)	123-131	2018

小島　肇	化学物質や医薬品などの安全性評価に用いる動物実験代替法の技術開発の現状と展望	イルシー Japan	136	23-31	2018
Kimura Y, Watanabe M, Suzuki N, Iwaki T, Yamakage K, Saito K, Nakajima Y, Fujimura C, Ohmiya Y, Omori T, Kojima H, Aiba S	The performance of an in vitro skin sensitisation test, IL-8 Luc assay (OECD442E), and the integrated approach with direct peptide reactive assay (DPRA)	J Toxicol Sci.	43(12)	741-749	2018
Kawai A, Goto T, Shibata T, Tani K, Mizutani S, Nishikawa A, Shibata T, Matsumoto S, Nagata K, Narukawa M, Matsui S, Ando M, Toguchida J, Monden M, Heike T, Kimura S, Ueda R	Current state of therapeutic development for rare cancers in Japan, and proposals for improvement	Cancer Sci.	109(5)	1731-1737	2018
Toyoda T, Cho YM, Akagi JI, Mizuta Y, Matsushita K, Nishikawa A, Imaida K, Ogawa K	A 13-week subchronic toxicity study of acetaminophen using anobese rat model	J. Toxicol. Sci.	43(7)	423-433	2018
Tsuchiya T, Kijima A, Ishii Y, Takasu S, Yokoo Y, Nishikawa A, Yanai T, Umemura T	Role of oxidative stress in the chemicalstructure-related genotoxicity of nitrofurantoin in Nrf2-deficient *gpt* delta mice	J. Toxicol. Pathol.	31(3)	169-178	2018
Tsuchiya T, Kijima A, Ishii Y, Takasu S, Yokoo Y, Nishikawa A, Yanai T, Umemura T	Mechanisms of oxidative stress-induced in vivo mutagenicity by potassium bromate and nitrofurantoin	J. Toxicol. Pathol.	31(3)	179-188	2018
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	92	2043-2054	2018

世戸孝樹，佐藤秀行，尾上誠良	医薬品の光安全性評価：光化学および薬物動態学の観点からのアプローチ	医薬品安全性学	3	81-92	2018
Van Viet E., Kuhnl J, Goebel C, Martinozzi-Teisier S, Alpee N, Ashikaga T, et al.	State-of-the-Art and New Options to Assess T Cell Activation by Skin Sensitizers: Cosmetics Europe Workshop	ALTEX	35 (2)	179-192	2018
Hoffmann S, Kleinstreuer N, Alepee N, Allen D, Marie Api A, Ashikaga T, et al.	Non-animal methods to predict skin sensitization (I): the Cosmetics Europe data base	Critical Reviews in Toxicology	48 (5)	344-358	2018
Kleinstreuer N, Hoffmann S, Alepee N, Allen D, Ashikaga T, et al.	Non-animal methods to predict skin sensitization (II): an assessment of defined approaches	Critical Reviews in Toxicology	48 (5)	359-374	2018
Hirota M, Ashikaga T, Kouzuki Y	Development of an artificial neural network model for risk assessment of skin sensitization using human cell line activation test, direct peptide reactivity assay, KeratinoSens™ and in silico structure alert parameter	Journal of Applied Toxicology	38 (4)	514-526	2018
Matsushita K, Takasu S, Kuroda K, Ishii Y, Kijima A, Ogawa K, Umemura T	Mechanisms underlying exacerbation of osmotic nephrosis caused by pre-existing kidney injury. Toxicological sciences	Toxicological sciences	165 (2)	420-430	2018
Matsushita K, Toyoda T, Morikawa T, Takahashi M, Inoue K, Ogawa K	A 13-week subchronic toxicity study of 2-ethylbutanal in F344 rats	Regulatory toxicology and pharmacology	100	118-126	2018
Toyoda T, Totsuka Y, Matsushita K, Morikawa T, Miyoshi N, Wakabayashi K, Ogawa K	γ-H2AX formation in the urinary bladder of rats treated with two norharman derivatives obtained from o-toluidine and aniline	Journal fo Applied Toxicology	38 (4)	537-543	2018

Matsumoto M, Furukawa M, Kobayashi K, Iso T, Igarashi T, Yamada T, Hirose A.	A 28-day repeated oral-dose toxicity study of insecticide synergist N-(2-ethylhexyl)-1-isopropyl-4-methylbicyclo[2.2.2] oct-5-ene-2,3-dicarbo-ximide in rats.	Fundam. Toxicol. Sci.	5	1-11	2018
Yamada T, Tanaka Y, Hasegawa R, Igarashi T, Hirose A.	Male-specific prolongation of prothombin time by industrial chemicals.	Fundam. Toxicol. Sci.	5	75-82	2018
Igarashi T, Serizawa H, Kobayashi K, Suzuki H, Matsumoto M, Iso T, Kawamura T, Inoue K, Ono A, Yamada T, Hirose A.	Initial hazard assessment of 4-benzylphenol, a structural analog of bisphenol F: Genotoxicity tests in vitro and a 28-day repeated-dose toxicity study in rats.	Regul. Toxicol. Pharmacol.	96	64-75	2018
Chesnut M, Yamada T, Adams T, Knight D, Kleinstreuer N, Kass G, Luechtefeld T, Hartung T.	Regulatory acceptance of read-across.	ALTEX	35	413-419	2018
Igarashi T, Takashima H, Takabe M, Suzuki H, Ushida K, Kawamura T, Matsumoto M, Iso T, Tanabe S, Inoue K, Ono A, Yamada T, Hirose A.	Initial hazard assessment of benzyl salicylate: In vitro genotoxicity test and combined repeated-dose and reproductive/developmental toxicity screening test in rats.	Regul. Toxicol. Pharmacol.	100	105-117	2018

平成31年3月28日

厚生労働大臣　殿

機関名　国立医薬品食[redacted]

所属研究機関長　職　名　所長

氏　名　奥田晴宏

次の職員の平成３０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）

（※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。

平成31年3月28日

厚生労働大臣　殿

機関名　国立医薬品食[redacted]

所属研究機関長　職　名　所長

氏　名　奥田晴宏

次の職員の平成３０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）　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

	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。

平成31年3月28日

厚生労働大臣　殿

機関名　国立医薬品食[redacted]

所属研究機関長　職　名　所長

氏　名　奥田晴宏

次の職員の平成30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)　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)

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。

平成31年3月28日

厚生労働大臣　殿

機関名　国立医薬品食[redacted]

所属研究機関長　職　名　所長

氏　名　奥田晴宏　[redacted]

次の職員の平成３０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）　病理部　室長

（氏名・フリガナ）　チョウ　ヨンマン　(チョウ　ヨンマン)

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。

平成３１年2月２１日

厚生労働大臣殿

機関名　東北大学

所属研究機関長　職　名　　総長

氏　名　大野　英男[redacted]

次の職員の平成３０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[redacted]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事項を示した　）

（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。

平成31年　4　月　5　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[redacted]
氏　名　髙崎　智彦

次の職員の平成３０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。

平成31年４月４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]

次の職員の平成３０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。

平成31年3月28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

次の職員の平成３０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。

平成31年3月28日

厚生労働大臣　殿

機関名　国立医薬品食[redacted]

所属研究機関長　職　名　所長

氏　名　奥田晴宏

次の職員の平成３０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　厚生労働行政推進調査事業

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）　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）

（※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。

平成31年3月28日

厚生労働大臣　殿

機関名　国立医薬品食[redacted]

所属研究機関長　職　名　所長

氏　名　奥田晴宏

次の職員の平成３０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）　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。

平成31年3月28日

厚生労働大臣　殿

機関名　国立医薬品食[redacted]

所属研究機関長　職　名　所長

氏　名　奥田晴宏

次の職員の平成３０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。

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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：　　）
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。