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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

光安全性試験の TG および光毒性 AOP の開発

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研究要旨

外因性光線過敏症は近年注目を集める有害事情の一つであり、本毒性リスク回避のために効果的な予測方法の開発が国内外で急務の課題となっている。本研究では *in vitro* 光化学的試験方法である ROS アッセイを主軸とした AOP を作成するため、光毒性物質の光生物化学的ならびに光化学的特性を精査することで光毒性反応機序のさらなる解明を行った。また、得られた科学的根拠をベースにした 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。

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A. 研究目的

近年、化合物の光安全性に対する関心の高まりから光毒性リスク評価に関する数多くの研究が行われている。ICH S10 で化合物の i) 光反応性および ii) 露光部位（皮膚や眼）への分布が光毒性発現に重要な因子として明記されている。当研究室では既に光化学的評価方法として reactive oxygen species (ROS) assay を開発し、本データと皮膚内動態情報の組み合わせることで信頼性ある光安全性評価が可能となることを明らかにした。この知見を検証すべく、本研究では ROS assay による光化学的特性および Franz 型拡散セルを用いた被験物質の *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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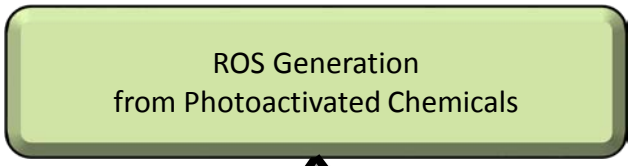
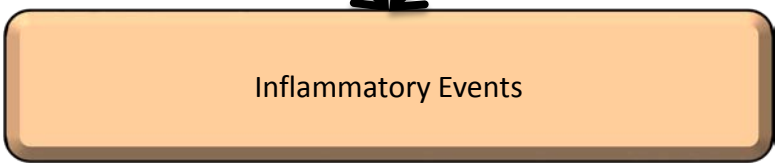
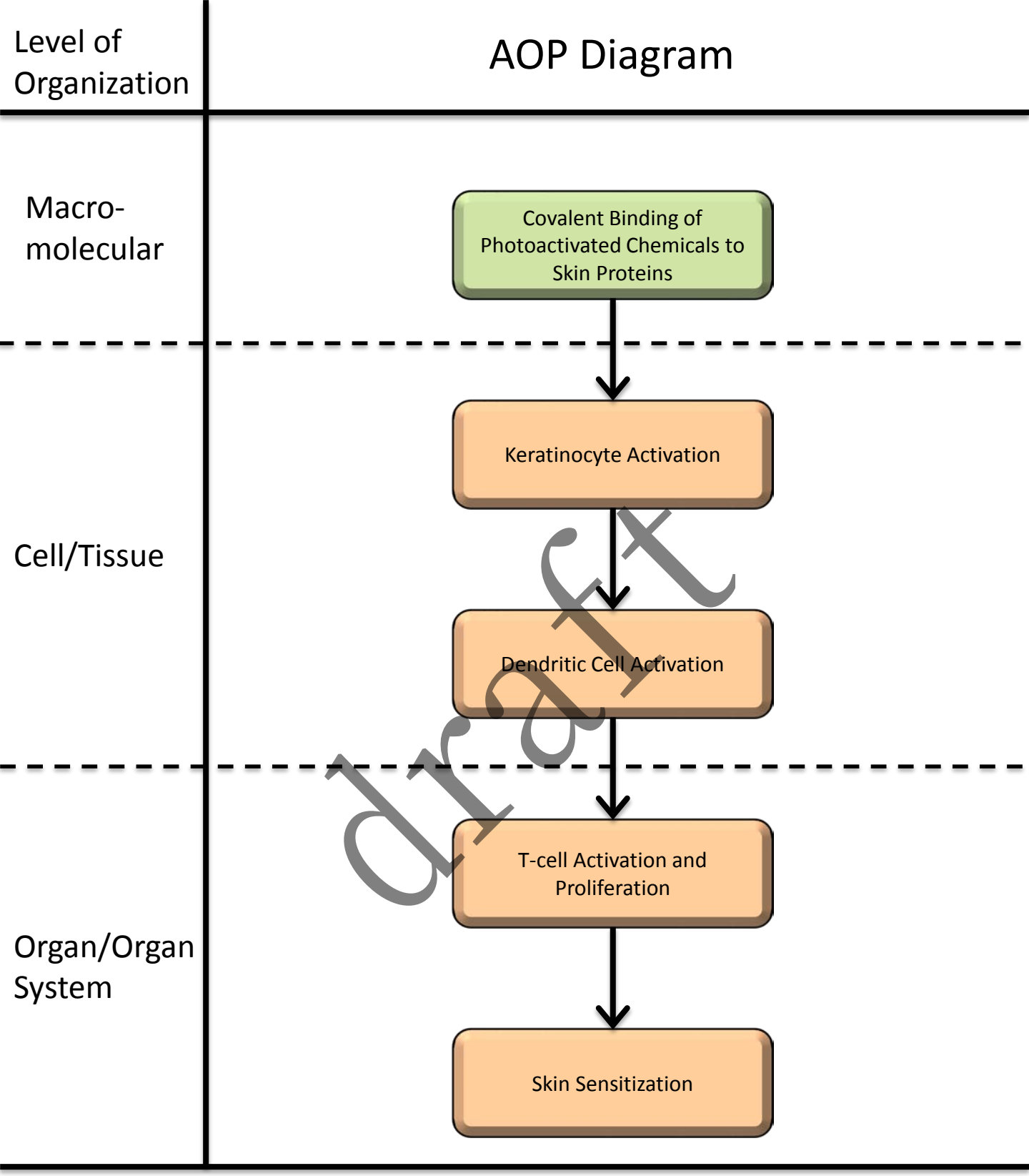
Level of Organization	AOP Diagram
Macro-molecular	
Cell/Tissue	
Organ/Organ System	


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

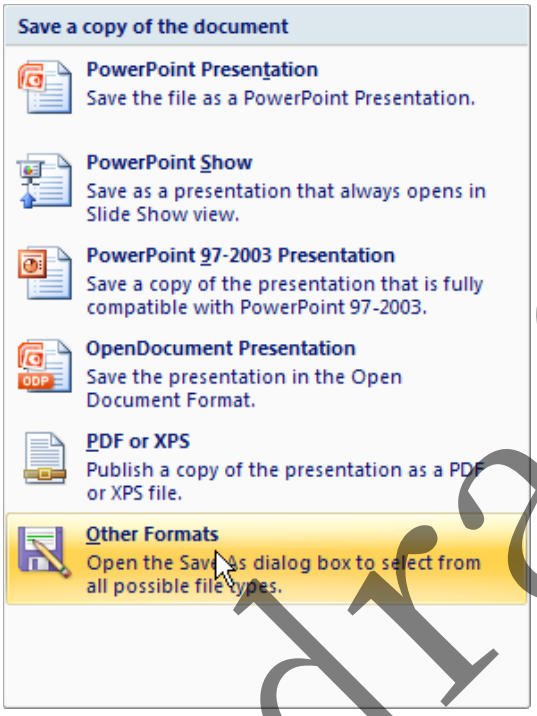


Fig 2

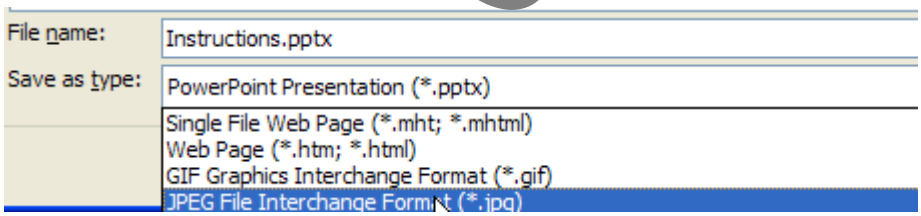


Fig 3