GUIDANCE DOCUMENT ON AN INTEGRATED APPROACH ON TESTING AND ASSESSMENT (IATA) FOR PHOTOTOXICITY

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List of Acrynonyms for IATA Phototoxicity

IATA:	integrated approach on testing and assessment
AOP:	adverse outcome pathway
MIE:	molecular initiating event
KE:	key event
MEC:	molar extinction coefficient
ROS:	reactive oxygen species
NRU:	neutral red uptake
PT:	phototoxicity
HOMO-LUMO:	energy gap between the highest occupied molecular orbital and the lowest unoccupied molecular orbital
MoA:	mode of action
IVIVC :	*in vitro/in vivo* correlations
TDS:	transdermal delivery systems
SARs:	structure-activity relationships
ADME:	absorption, distribution, metabolism and excretion
PSA:	polar surface area
PBPK:	physiologically-Based pharmacokinetic
RNO:	*p*-nitrosodimethyl aniline
NBT:	nitroblue tetrazolium
PIF:	photoirritancy Factor
MPE:	mean photo effect
ECVAM:	the European centre for the validation of alternative methods
ESAC:	the EURL ECVAM scientific advisory committee
RhE PT:	reconstructed human epidermis phototoxicity method

SUMMARY

1. A number of efforts have been made to establish approaches to testing and assessment to clarify photosafety of test chemicals, and combination use of these data (e.g. *in silico* predictions, *in chemico*, *in vitro*, *in vivo* data) would be efficacious for more reliable photosafety evaluation. This document has two aims; (i) it suggests an integrated approach on testing and assessment (IATA) for photoirritation hazard identification, and (ii) to provide key information characteristics of each of the individual information sources comprising the IATA. Furthermore, it provides guidance on how and when to integrate existing and/or newly generated information for decision making, including decisions on the need for further testing or final decisions on classification and labelling regarding the potential phototoxic effects of test chemicals.

INTRODUCTION AND SCOPE

2. Phototoxic skin responses after exposure to photoreactive chemicals have been recognized as undesirable side effects, and several classes of chemicals, even when not toxic by themselves, may become reactive under exposure to environmental light, inducing undesired phototoxic skin responses [1-3].

Phototoxic reactions can be categorized as photoirritant, photogenotoxic or photoallergic, and some chemicals can cause all three types of reactions [4]. In a clinical evaluation, a systematic approach including pertinent history, physical examination, phototesting, photopatch testing, and laboratory investigation are essential steps in evaluating a photosensitive patient [5]. In addition, evaluating the phototoxic potential of a chemical is necessary at the early phase of product development to minimize unwanted reactions in humans. Therefore, a number of efforts have been made to design a model system for the assessment of photosensitive/phototoxic potential through analytical and biochemical methods [6-15]. Although multiple photoirritation testing tools have been developed and validated so far, there are no validated test methods (i.e., OECD Test Guideline) to evaluate photosensitive or photogenotoxic potential of chemicals to this date. While IATA can be explored using non-validated test methods and certainly be useful for endpoints with scarcity of test methods, such IATA would be difficult to use in regulatory contexts. Therefore, the Adverse Outcome Pathway (AOP) and IATA contained in the present Guidance Document is relevant only for photoirritation of chemicals for which several OECD test guidelines exist. Guidance on biologicals (i.e., peptides, proteins) or pharmaceuticals may be consulted elsewhere [17].

3. There is a general agreement on the key chemical and biological events underlying phototoxic skin responses [2, 3, 16, 17], and this knowledge can be summarized in an AOP. Figure 1 shows an AOP that identifies a pre-molecular initiating event (pre-MIE), a molecular initiating event (MIE), and a key event (KE) leading to the adverse outcome, phototoxicity.

4. When a chemical absorbs a photon energy (pre-MIE), electrons can be promoted from occupied orbitals (the ground state) to an unoccupied orbital (S_1, S_2), depending upon the bond type and associated energy level. Unpaired singlet state electrons (opposite spin) may be converted to a triplet state (parallel spin) by inversion of the spin via intersystem crossing of the absorbed energy. Absorption of sunlight by phototoxins or phototoxic chemicals, followed by photochemical reaction, is considered to be a key trigger for phototoxicity [18], because photo-excited chemicals may react with biomolecules, leading to phototoxic events [2, 19]. In this context, the UV-absorbing property of chemicals can be a potential indicator for phototoxic risk. Henry and co-workers demonstrated that chemicals with a molar extinction coefficient (MEC) of less than 1,000 $M^{-1}cm^{-1}$ at any wavelength between 290 – 700 nM showed low phototoxic risk [20], and therefore this threshold can be used to differentiate compounds that require consideration from those that do not.

5. Absorbed energy can be dissipated by internal conversion, fluorescence (from a singlet state), phosphorescence (from a triplet state) or via chemical reaction, giving rise to photoproducts and/or intermediates that are potentially reactive with other molecules, including various biomolecules (MIE). 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) lies at a comparatively low energy. Energy transfer from an excited triplet photosensitizer to oxygen (type II photochemical reaction) could produce excited singlet oxygen which might, in turn, participate in oxidation of membrane lipids and proteins, or induce DNA damage. Electron or hydrogen transfer could lead to the formation of free radical species (type I

photochemical reaction) that can react with biomolecules either directly or in the presence of oxygen, forming secondary free radicals such as peroxyl radicals or the very reactive hydroxyl radical, a known intermediate in the oxidative damage of DNA and other biomolecules. These direct and/or indirect photochemical reactions of excited photosensitizers may lead to the following adverse outcomes (AO): (i) photoirritation through oxidative damage to cellular lipids and proteins, (ii) photogenotoxicity through DNA damage, and (iii) photoallergy through formation of photoantigens [4, 13], via the identified Key Events (Fig.1). These phototoxic reactions could be induced simultaneously by some chemicals, and the photochemical reaction of some compounds with reactive oxygen species (ROS) could also result in the yield of toxic degradants [4, 21].

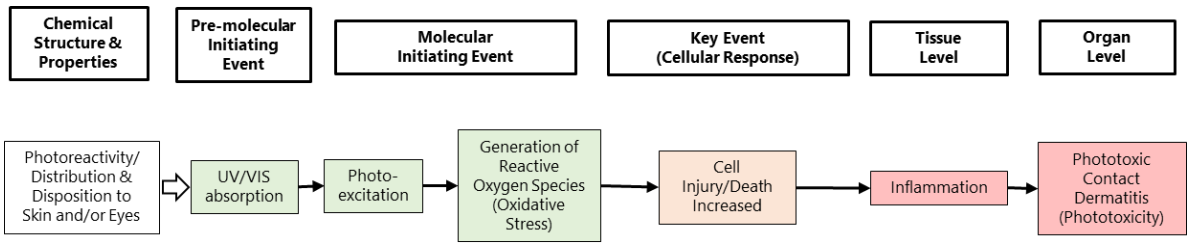

Figure 1: Flow diagram of the adverse outcome pathway and the intermediate steps associated with phototoxic responses.

6. Knowledge of the phototoxicity pathway has prompted development of *in silico*, *in chemico*, and *in vitro* methods, addressing specific MIE and KEs in the

AOP and providing alternative to *in vivo* methods for assessment of phototoxicity. Information generated by these methods can contribute to the assessment of the phototoxic potential of chemicals when used as information sources within defined approaches and IATA. Within such AOP-informed defined approaches/IATA, the different information sources would target MIE and KEs along the defined toxicity pathway and the results could be used to inform a regulatory decision.

7. It is noted that there is no validated *in vivo* method (i.e., OECD Test Guideline) for evaluating phototoxicity of a chemical, but there are a few non-standardized models available [3, 22, 23]. Diagnosis of phototoxicity can be made clinically by photopatch testing [6], and skin biopsy can also help elucidate the diagnosis. The photopatch test consists of topical application of non-irritating doses of potential phototoxins or phototoxic chemicals in duplicate, and exposing one area to a UVA/UVB lamp while keeping the other covered.

8. A number of validated in *vitro/in chemico* methods for screening phototoxic chemicals exist, namely OECD TG 101 on UV-VIS absorption spectra [20, 24], OECD TG 498 on In Vitro Phototoxicity – Reconstructed Human Epidermis Phototoxicity Method (RhE PT) [25], OECD TG 432 on In Vitro 3T3 Neutral Red Uptake (NRU) Phototoxicity Test [12], and OECD TG 495 on Reactive Oxygen Species (ROS) Assay for Photoreactivity [26]. The ROS assay has been also included by the ICH S10 guideline [17] as an optional initial in chemico screening tool for evaluating the photoreactivity of pharmaceuticals.

9. It is important to note that the in vitro/in chemico assays in OECD Test Guidelines exhibit limitations. For example, the outcomes from the ROS assay were

not always indicative of phototoxic potential since photolabile chemicals could also be captured. Alternatively, 3T3 NRU PT may be used, but this method could lead to false-negative predictions for chemicals predominantly absorbing in the UVB range, since only a UVA light source is used in the assay to avoid the cytotoxic effect of UVB light on 3T3 cells [27]. Although RhE PT commonly utilizes the UVA/VIS, some studies confirm that the RhE tissues can also tolerate UVB under controlled conditions [25]. This might be an advantage compared to most of the cell-line based assays that do not tolerate the UVB. Based upon these assay limitations, a combination of available assay systems might be needed for more reliable photosafety evaluation and supporting the development of IATA.

MAPPING OF INFORMATION SOURCES THAT CAN BE USED WITHIN DEFINED APPROACHES OR IATA FOR PHOTOTOXICITY BY APPLYING THE AOP AS A FRAMEWORK

10. Various assessment tools for evaluating phototoxic potential of chemicals have been developed based on the pathogenetic mechanisms of phototoxicity. Of interest are tools that include *in silico* prediction systems [28-30] for deductive estimation of hazard from existing knowledge (DEREK) and from the energy gap between the highest occupied molecular orbital and the lowest unoccupied molecular orbital (HOMO-LUMO gap). DEREK allows toxicity prediction of chemicals based on structures known to be associated with the incidence of toxicity

[28], and HOMO-LUMO gap evaluation provides a measure of the photoreactive potential of chemicals [29, 31]. The OECD QSAR Toolbox has traditionally been a decision-support system incorporating information and data from various sources into a single framework. In addition to the structural information from the *in silico* tools, the photochemical properties of the test chemicals are also a key indicator of the phototoxic potential: for example, UV spectral analysis can provide reliable information on the photoexcitability of chemicals [20]. ROS assay and micellar ROS (mROS) assay were also proposed for evaluating the phototoxic risk of chemicals, since photo-activated phototoxins or phototoxic chemicals typically generate ROS, such as singlet oxygen and superoxide [13, 32].

11. The assessment of phototoxic potential can include the evaluation of exposure parameters, understanding of dermal and ocular bioavailability, information on pre-MIE/MIE and KEs and any other supporting information, i.e. information from non-testing and regulatory testing methods, including those designed to address other health or environmental endpoints that may inform phototoxicity assessment. The elements and information sources that could be used within defined approaches or IATA for phototoxicity assessment are listed in Table 1. Note that this is not an exhaustive list and does not imply any judgement about the suitability of any of the individual tests listed for a specific assessment. It has to be noted that the elements addressed within a specific defined approach or IATA and the type of information sources used to populate each individual element may vary depending on the scope and the specific regulatory requirement. For example, certain regulatory purposes (e.g. hazard identification) may require fewer elements for the assessment than for

more complex regulatory needs (e.g. risk assessment). It is therefore envisaged that different defined approaches and IATA solutions may be possible depending on the chemical under investigation, the regulatory need and the specific regulatory requirements in the different regions.

Table 1. Elements and examples of information sources that can be used within defined approaches and IATAs for phototoxicity

Elements	Information sources addressing each element	Validation status/weight of importance
Exposure consideration	● Applied dose	– / mid-high
	● Frequency of dosing	– / mid
	● Formulation effects	– / mid-high
	● Route of exposure	– / mid-high
	● Accumulation of compounds in the skin/eyes	– / high
	● *In vitro* to *in vivo* extrapolation	– / mid-high
Chemical descriptors	**Chemical structure**	
	● Structure alert [28]	– / mid-high
	● QSAR model [30]	– / mid-high
	Physicochemical properties	
	● Molecular weight	– / mid
	● pK_a	– / low
	● Partition coefficient (log *P*, log *D*)	– / mid-high
	● Water solubility	– / low
	● *in vitro* membrane permeability [33]	Validated / high
Skin penetration	**Non-testing methods**	
	● Characterization of skin absorption with use of physiologically-based pharmacokinetic models [34]	Validated / high
	Testing methods	
	● OECD TG 427 (Skin absorption: *in vivo* methods) [35]	Validated / high

	• OECD TG 428 (Skin absorption: *in vitro* methods) [36]	Validated / high
AOP Pre-MIE: Photoexcitation	• UV/VIS absorption [20, 24] • OECD TG 495 (ROS assay) • Photostability testing [20] • Homo-Lumo gap calculation [29]	Validated / high Validated / high – / low-mid – / low
AOP MIE: Oxidative stress	• Photohemolysis model [8] • Oxygen consumption in *Bacillus subtilis* [9] • Yeast growth inhibition assay [37] • DNA photocleavage assay [15]	– / low-mid – / low – / low-mid – / low/mid
SARAOP key event: Cell injury/death increased	• OECD TG432 (3T3 NRU phototoxicity testing) [12] • OECD TG498 (*in vitro* reconstructed human epidermis phototoxicity test)	Validated / high Validated / high

DESCRIPTION OF THE ELEMENTS OF THE IATA FOR PHOTOTOXICITY

Exposure consideration

12. The target organs for phototoxicity are skin and eyes which are exposed to light. Systemically or topically available concentrations of test chemical can be predicted in different body compartments and relevant target organs are identified for further assessment according to the predicted concentrations. This information can contribute to formulating the Mode of Action (MoA) hypothesis together with results from the *in silico* and *in vitro* profiles. The concentration range simulated for a target organ can be further used to predict the photosafety of tested chemicals. In addition to the primary route of exposure as the primary route of exposure, considerations for other routes of exposure have to be taken into account, e.g. inhalation and oral uptake.

An *in vitro* model that exhibits *in vitro/in vivo* correlations (IVIVC) is also a powerful tool since it can efficiently predict product performance *in vivo*. While the concept of IVIVC has been utilized mostly for oral dosage forms, demonstrations of IVIVC with *in vitro* permeation testing (IVPT) for transdermal delivery systems (TDS) are emerging [38]. These approaches would also provide prediction of toxicokineticbehavior of tested chemicals in the photosafety assessment.

Chemical descriptors

13. Computer-based assessment of potential toxicity has become increasingly popular in recent years, leading to reduced number of animals used in toxicity testing. Structure-activity relationships (SARs) can be used to predict human health hazards and, as such, may be of use in chemical testing strategies [28]. The OECD QSAR Toolbox is a software designed to support hazard assessment as well as to increase mechanistic and other knowledge on chemicals. The OECD QSAR Toolbox has traditionally been a decision-support system incorporating information and data from various sources into a single framework. The knowledge-based system DEREK was also developed under the guidance of a multinational collaboration of expert toxicologists and it provides a qualitative approach to toxicity prediction. Major developments of the DEREK program and other knowledge-based tools have taken place, and they are currently available for phototoxic prediction [30]. Since the defined chemical space for phototoxicity is limited, careful consideration should be made for phototoxicity prediction on the new chemical structure.

14. When assessing phototoxicity of a test chemical in systemic and/or topical exposure, one of the most critical factors is lipophilicity, because it governs the passive membrane partitioning. Measured/calculated lipophilicity metrics are often utilized to predict absorption of test chemical. The parameter that determines the lipophilicity of a molecule is log*P* (the partition coefficient of the molecule between an aqueous and lipophilic phase, usually water and octanol). While the partition coefficient is used to calculate properties such as membrane permeability and water solubility, it also has importance in the prediction of biological activities, absorption, distribution, metabolism and excretion (ADME), and toxicological endpoints. In addition, it is evident from an examination of experimental data that polar surface area (PSA) and the molecular volume components, as well as volatility or evaporation, may also affect dermal bioavailability.

Skin penetration

15. For better understanding on phototoxic potential of a test chemical, skin absorption of dermally-applied chemical, and to a lesser extent penetration, and/or distribution to the skin after systemic administration may be key determinant. Theoretically, for dermally-applied chemicals, a compound cannot induce phototoxic symptoms in deeper layers of the epidermis unless it is absorbed and penetrates the upper layers first. The epidermis, in particular the *stratum corneum* (i.e. the dead keratinized cells of the epidermis), represents the most important barrier in dermal uptake. Hence, bioavailability is often considered in the context of penetration of the *stratum corneum*. Considerable effort has been directed toward quantifying

penetration across *stratum corneum* of the skin and in estimating the steady-state adsorption of organic materials applied to the skin as aqueous solutions [39]. Skin transport occurs via passive diffusion in response to the concentration gradient between the application surface and the epidermal-dermal interface.

16. So far, both *in vivo* and *in vitro* OECD Test Guidelines have been adopted for identifying dermal toxicokinetic behavior [35, 36]. The *in vivo* method for determining the penetration of a substance through the skin of an animal and into the systemic compartment is described in OECD TG 427 [35]. The *in vitro* method in OECD TG 428 [34] is based on the permeability of a test substance from its formulation applied as a finite dose across human or animal skin preparations. In addition to the variation between different sources of skin and the acceptor fluid, homogeneous application of the test material, seals and stirring speed amongst others can be critical parameters for reproducibility.

17. Physiologically-Based pharmacokinetic (PBPK) models mathematically describe interconnected compartments representing the human body, considering ADME properties of a chemical within the organism [34]. These models facilitate extrapolations, i.e. predict concentrations in different compartments, across studies, species, routes and dose levels. Dermal PBPK models describe the skin permeation and disposition of the test chemical following the application of a dermatological product on the skin of virtual healthy and diseased human subjects.

AOP Pre-MIE: Photoexcitation

18. Photoreactivity of test chemical can be assessed by *in chemico* testing systems such as UV/VIS absorption [20, 24] and ROS assay [13], and these testing approaches were already adopted by ICH S10 and OECD guidelines. Combination of photostability data and other suitable parameter might be partly used as a screening tool to determine early photosafety risks of test chemicals, although photostability data alone was reported to be less effective for reliable photosafety prediction [20].

19. As an *in silico* approach for photosafety prediction, Homo-Lumo gap calculation was also reported for the evaluation of the phototoxic potential of a virtual compound before it has been synthesized [29]. In theory, the hardness of a molecule is defined as the gap in energies between the Homo and Lumo frontier orbitals, and the most stable structure should have the largest Homo-Lumo energy gap. Since phototoxicity requires activation of a molecule by UV/VIS light, the magnitude of a molecule's Homo-Lumo gap have some relationship to the potential for phototoxicity [40].

UV/VIS absorption	
General Description	
Regulatory use	Identification on photoexcitability of test chemicals by spectroscopic determination of UV/VIS-absorbing properties
Validation & regulatory acceptance status	Validated and adopted as OECD TG 101; presented under guidance document ICH S10.

Potential role in the IATA	Absorption of sunlight by phototoxins or phototoxic chemicals, followed by photochemical reaction, is considered to be a key trigger for phototoxicity [18], because photo-excited chemicals may react with biomolecules, leading to phototoxic events [2, 19]. In this context, the UV-absorbing property of chemicals can be a potential indicator for phototoxic risk, and Henry, et al. demonstrated that chemicals with a molar extinction coefficient (MEC) of less than 1,000 $M^{-1}cm^{-1}$ showed low phototoxic risk [20].
Description	Each chemical is dissolved in distilled water or appropriate organic solvent at several concentrations (e.g., 0.001, 0.01 and 0.1 μM), and the final concentration can be reduced if the tested chemical is found to be an intense UV/VIS absorber. UV/VIS absorption spectra (290–700 nm) are recorded with a spectrophotometer interfaced to a PC for data processing. MEC values can be calculated from maximum absorbance at several concentrations.
Scientific basis including MoA	When a chemical absorbs photon energy, electrons can be promoted from occupied orbitals (the ground state) to an unoccupied orbital (S_1, S_2), depending upon the bond type and associated energy level. Unpaired singlet state electrons (opposite spin) may be converted to a triplet state (parallel spin) by inversion of the spin via intersystem crossing of the absorbed energy. Absorbed energy can be dissipated by internal conversion, fluorescence (from a singlet state), phosphorescence (from a triplet state) or via chemical reaction, giving rise to photoproducts and/or intermediates that are potentially reactive with other molecules, including various biomolecules, potentially leading to various phototoxic symptoms.

Protocol available	Experimental protocol was established by Henry, et al. [20] and Bauer, et al. [24].
Strengths and weakness	Strengths - This *in chemico* test method offers rapid, reproducible and high-throughput (i.e., using 96-well method approaches) results. - Test chemicals that do not show significant absorbance (e.g. MEC >1000 M-1cm-1) may not need further photosafety evaluation Weakness - Some chemicals can be UV/visible light absorbers but do not pose phototoxicity hazard or risk, 'positive' prediction from this method needs to be further evaluated with subsequent testing methods [13]. - Standardized conditions for determination of the MECs are critical. Selection of an adequate solvent is driven by both analytical requirements (e.g., dissolving power, UV-visible light transparency) and physiological relevance (e.g., pH 7.4-buffered aqueous conditions).
Applicability domain and limitations	Applicability - The test method is applicable to substances. Limitations - It may not be possible to evaluate poorly-water soluble chemicals in this *in chemico* test method. - The limitations of the chosen method need to be considered (e.g., linear range of the experimental set up). Potential artifacts (e.g., due to concentrations that are too high or precipitating) has to be carefully assessed.

	- For calculation of MEC, defined molecular weight of test chemical is needed, so that it is challenging to apply this test method to complex materials/chemical without defined molecular weight.
Predictive capacity	Henry, et al. demonstrated that all 35 phototoxins or phototoxic chemicals tested had absorbance intensities significantly above an MEC threshold of 1,000 L mol^{-1} cm^{-1} [20]. Bauer, et al. verified the predictive performance of MEC threshold (1,000 L mol^{-1} cm^{-1}) with 76 chemicals [24]. Onoue, et al. also demonstrated that the MEC threshold (1,000 L mol^{-1} cm^{-1}) could be mostly effective for photosafety testing on 51 chemicals (33 cosmetics and 18 non-cosmetics) [41].
Reliability	When measuring MEC values of 76 chemicals in 6 laboratories, all chemicals were found to have agreement of classification between laboratories [24].

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ROS assay	
General Description	
Regulatory use	Identification of photoreactivity of test chemicals by determination of ROS generation from irradiated chemicals
Validation&regulatory acceptance status	Validated and adopted as OECD TG495; presented under Guidance Document ICH S10.
Potential role in the IATA	The primary event in any photosensitization process is the absorption of photons of the appropriate wavelength, which allows a chromophore to reach an excited state. The excitation energy is often transferred to oxygen molecules, followed by generation of ROS: superoxide

	through type I reaction and singlet oxygen through type II reaction by photo-excited molecules. These appear to be the principal intermediate species in the phototoxic response [13, 42]. The ROS assay can monitor generation of ROS, such as singlet oxygen and superoxide, from photoirradiated chemicals; therefore, the ROS data can be used to evaluate the photoreactivity of chemicals [13, 43].
Description	In the ROS assay, generation of singlet oxygen was detected by spectrophotometric measurement of *p*-nitrosodimethyl aniline (RNO) bleaching, followed by decreased absorbance of RNO at 440 nm. Although singlet oxygen does not react chemically with RNO, the RNO bleaching is a consequence of singlet oxygen capture by the imidazole ring, resulting in the formation of a trans-annular peroxide intermediate capable of inducing the bleaching of RNO as follows; Singlet oxygen + Imidazole → [Peroxide intermediate] → Oxidized imidazole [Peroxide intermediate] + RNO → RNO + Products The generation of superoxide could be determined by the reduction of nitroblue tetrazolium (NBT) as indicated below; NBT can be reduced by superoxide anion via a one-electron transfer reaction, yielding partially reduced (2 e^-) monoformazan (NBT^+) as a stable intermediate. Thus, superoxide can reduce NBT to NBT^+, whose formation can be monitored spectrophotometrically at 560 nm. Superoxide + NBT → O_2 + NBT^+

Scientific basis including MoA	In any type of phototoxic event, penetration and absorption of light in the skin, eyes, or other UV-exposed tissues can be a critical factor for triggering phototoxic cascades, and the absorption of photon energy by the phototoxin results in excitation of the molecule itself [18]. Since molecular oxygen can act as the predominant acceptor of excitation energy, energy can be transferred from photo-excited chemicals to oxygen through type II photochemical reaction, resulting in the generation of singlet oxygen. Transfer of an electron or hydrogen could also lead to the formation of free radical species such as superoxide, peroxyl radicals or reactive hydroxyl radical through a type I photochemical reaction. Thus, photo-excitation of chemicals tends to produce ROS, which may be one of major causative agents of phototoxic events.
Protocol available	OECD TG495 [26]
Strengths and weakness	<u>Strengths</u> - This *in chemico* test method offers rapid and reproducible photosafety prediction [44-46]. - For this test method, UVB light source can be used, that is usually excluded in the cell-based photosafety testing. <u>Weakness</u> - To avoid spectral interference of discoloring chemicals in ROS determination, an experimental control has to be employed upon exposure of tested chemical alone to simulated sunlight, to subtract control readings from sample readings.

	- In theory, the ROS assay can provide highly sensitive predictions (i.e., false positives), since it may capture all photochemically active substances [13]. Some photolabile substances would be judged as positive in the ROS assay if they are potent ROS generators in their photodegradation pathways.
Applicability domain and limitations	Applicability - The test method is applicable to substances. Limitations - The poorly-water soluble chemicals might be untestable by this *in chemico* test method. In such a case, the mROS assay is available partly [14, 32]. In the mROS assay, Tween20 is added to solvent system, and the formed micelle can enhance the solubility of most test chemicals. However, mROS assay has not been formally validated yet. - The chemicals with potent chromophores (e.g., rose bengal) might be untestable because of spectral interference.
Predictive capacity	The validation study was previously undertaken to verify the applicability of different solar simulators and assay performance [44, 45]. In 7 participating laboratories, 2 standards and 42 coded chemicals, including 23 phototoxins or phototoxic chemicals and 19 non-phototoxic drugs/chemicals, were assessed by the ROS assay using two different solar simulators (ss-1 and -2). In both solar simulators, the intra- and inter-day precisions (coefficient of variation; CV) for quinine were found to be below 10%. The inter-laboratory CV for

	quinine averaged 15.4% (ss-1) and 13.2% (ss-2) for singlet oxygen and 17.0% (ss-1) and 7.1% (ss-2) for superoxide, suggesting high inter-laboratory reproducibility even though different solar simulators were employed for the ROS assay. In the ROS assay on 42 coded chemicals, some chemicals (ca. 19–29%) were unevaluable because of limited solubility and spectral interference. Although several false positives appeared with positive predictivity of ca. 76–92% (ss-1) and ca. 75–84% (ss-2), there were no false negative predictions in both solar simulators.
Reliability	Multi-center validation study on the ROS assay demonstrated satisfactory transferability, accuracy, precision, and predictivity, as well as the availability of other solar simulators [44, 45].

AOP MIE: Oxidative stress

20. Oxidative stress is the consequence of an imbalance between ROS and the failure of antioxidants to neutralize excessive ROS production. Oxidative stress sometimes induces alterations in proteins, lipid peroxidation, DNA damage and apoptotic cell death, so oxidative stress by irradiated test chemical can be measured indirectly by several testing approaches. The photohemolysis model was proposed to clarify the ability of test chemical to induce colloid-osmotic photohemolysis of erythrocytes [8]. After irradiation with UVA/VIS, the permeability of the cytoplasmic membranes to cations is enhanced, which leads to swelling and osmotic lysis of the erythrocytes. Photohemolysis is a delayed process, and it develops during minutes or even hours after irradiation. The hemolysis curve has a sigmoid

shape, and no threshold dose is observed. The hemolysis rate parameter is used for the quantitative characterization of the hemolysis curves, defined as the reciprocal value of the time of postirradiative incubation during which 50% of the cells are lysed. However, the photohemolysis model may provide false-negative predictions on some phototoxins or phototoxic chemicals when the mechanism of phototoxicity is not related to cellular membrane damage [37]. Yeast growth inhibition assay using *Candida albicans* is available for detection of the damage to DNA and/or cell organelles [37]. A previous study demonstrated that yeast growth inhibition assay could predict the phototoxic potential of psoralens correctly that were judged as negative in the photohemolysis model [37]. Oxygen consumption in *Bacillus subtilis* can also be indicative of the photosensitizing ability of test chemical [9], the principle of which is based on the analysis of variations in the consumption of oxygen by *Bacillus subtilis* as measured by Warburg's apparatus or an oxygenometric cell. The DNA photocleaving assay using capillary gel electrophoresis was designed to predict the phototoxic potential of test chemical with the use of pBR322 DNA, a plasmid DNA [15]. Generally, chromosome aberrations and DNA strand breakage are characteristic types of genetic damage induced by phototoxins or phototoxic chemicals; therefore, strand break activity was evaluated using supercoiled plasmid DNA, a very sensitive tool for damage detection that was monitored by capillary gel electrophoretic analysis.

AOP key event: Cell injury/death increased

21. The 3T3 NRU PT is designed to detect phototoxicity induced by the combined action of a chemical and attenuated UVA/visible light by using an *in vitro* cytotoxicity assay in the Balb/c 3T3 mouse fibroblast cell line [12]. The *in vitro* 3T3 NRU PT is a highly sensitive methodology for evaluating phototoxicity potential. In addition, the human reconstituted epidermis (RhE) model has been thought as a suitable 3-D *in vitro* tool to evaluate phototoxicity potential of test chemicals intended for topical use [25], and may be considered more relevant for human hazard identification

3T3 NRU phototoxicity testing	
General Description	
Regulatory use	Identification of phototoxicity potential of test chemicals using Balb/c 3T3 cultures
Validation&regulatory acceptance status	Validated and adopted as OECD TG 432; presented in ICH S10 guidance document
Potential role in the IATA	The 3T3 NRU PT assesses the cytotoxic effect of a test substance after exposure to a non-cytotoxic dose of UVA light compared with that in the absence of exposure, and the cytotoxicity is expressed as a concentration-dependent reduction of the uptake of the vital dye. Chemicals identified as positive in this test may be phototoxic *in vivo*, following topical application or systemic application and distribution to the UV-exposed tissues.

Description	For irradiation, UV filters were installed with solar simulator to attenuate wavelengths below 320 nm partly, and the ratio of UVB to UVA can be adjusted by filter and light source to optimize conditions to detect UVB-induced phototoxicity in this assay while minimizing cytotoxicity. The cells are exposed to a test chemical in the presence (+Irr) (dose of 5 J/cm^2 of UVA) or absence (-Irr) of UVA light, and viability is assessed 24 hours later by spectrophotometric measurement of neutral red dye uptake by the compound treated cells compared to vehicle treated controls. Chlorpromazine is used as positive control, while Earle's Balanced Salt Solution or other buffered solution may be used as negative controls. The concentration of test article causing a 50% reduction in neutral red dye uptake (IC_{50}) reflects cytotoxic potential. The phototoxic potential is also expressed through the use of two different indices: Photoirritancy Factor (PIF) and Mean Photo Effect (MPE). The PIF is determined by comparing the IC_{50} +Irr to the IC_{50} -Irr and by definition is only useful when IC_{50} values can be determined both with and without UVA exposure. The MPE is determined by comparing the two concentration response curves (-Irr and +Irr) over the range of active test article doses. With respect to phototoxicity prediction on the basis of the results from 3T3 NRU PT, three cases may be considered: 1) a test article with a PIF <2 or an MPE <0.1 predicts "no phototoxicity"; 2) a test article with a PIF >2 and <5 or an MPE >0.1 and <0.15 predicts "equivocal phototoxicity"; and 3) a test article with a PIF >5 or an MPE >0.15 predicts "phototoxicity" [47].

Scientific basis including MoA	The 3T3 NRU PT is conducted using Balb/c 3T3 mouse fibroblasts to assess the phototoxicity potential of a test article. The assay quantitatively determines the photo-cytotoxic potential of a test article by comparing the reduction in neutral red dye uptake in Balb/c 3T3 cultures exposed to the serial dilutions of a test article, to the neutral red dye uptake in control (the test article vehicle). Phototoxins or phototoxic chemicals can induce cell damage through formation of ROS and other mechanisms that lead to increased permeability of the lysosomal membrane, reduction in the pH gradient, and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of neutral red dye. It is thus possible to distinguish between viable and damaged or dead cells.
Protocol available	OECD TG432 [12, 48]
Strengths and weakness	<u>Strengths</u> - The assay quantitatively determines the cytotoxic potential of a test chemical. - High throughput assay; can screen large numbers of test chemicals for phototoxicity potential - High negative predictivity (further photosafety testing is generally not warranted for test chemicals which are not predicted to have phototoxicity potential in this test method) <u>Weakness</u> - Highly sensitive assay. Detection level is far more sensitive than the magnitude of biological effect.

	- In the 3T3 NRU PT, a UVA light source with filter to attenuate UVB is used since 3T3 cells are not tolerant to higher doses of UVB light, so the 3T3 NRU PT may provide false-negative prediction for chemicals predominantly or solely absorbing in the UVB range [27].
Applicability domain and limitations	Applicability - The test method is applicable to substances and mixtures. Limitations - The poorly-water soluble chemicals might be untestable. - In the 3T3 NRU PT, UVB radiation is generally attenuated since it causes cell death by UVB radiation; therefore, chemicals excited by only UVB exposure produce false-negative results in the assay. UVB-induced phototoxicity is rarely a problem for a compound with systemic exposure only, but is more relevant with topical exposure and information of route of exposure and distribution should therefore be taken into account in the selection of *in vitro* method.
Predictive capacity	20 chemicals were tested in the pre-validation phase whereas 30 chemicals were tested in the validation of 3T3 NRU PT [49]. An almost perfect correlation of in vitro versus in vivo results was obtained (between 95% and 100%), when either PIF or MPE were used to predict the phototoxic potential.
Reliability	The 3T3 NRU PT was developed and validated under the auspices of ECVAM from 1992–1997, to establish a

	valid *in vitro* alternative to the various *in vivo* tests in use [50]. A second validation study was also carried out in 1997 to evaluate the method specifically in terms of selected UV filter chemicals. ESAC subsequently endorsed the validity of the test with respect to these chemicals.

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in vitro reconstructed human epidermis phototoxicity test	
General Description	
Regulatory use	Identification of phototoxic potential of test chemicals using reconstructed human epidermis phototoxicity test (RhE PT)
Validation & regulatory acceptance status	Validated and adopted as OECD TG498; also presented in guidance document ICH S10
Potential role in the IATA	The *in vitro* RhE PT can be used to identify the phototoxic potential of a test chemical after topical application in RhE tissues in the presence and absence of simulated sunlight. Phototoxicity potential is evaluated by the relative reduction in viability of cells exposed to the test chemical in the presence as compared to the absence of simulated sunlight. Chemicals identified as positive in this test may be phototoxic *in vivo* following topical application to the skin, eyes, and other external light-exposed epithelia. Complementary to cell monolayer phototoxicity tests, this 3-D model allows the topical application of a large panel of chemicals with different physicochemical properties as water insoluble or extreme pH values chemicals, finished products or complex formulations.

Description	Several concentrations of test chemical prepared in a solvent are applied topically to RhE tissues and incubated at standard culture conditions for 18 to 24 hours to allow penetration into the living tissue. A positive control (e.g., chlorpromazine) and appropriate solvent controls are also applied topically to RhE tissues and tested in parallel. Half of the tissues in each treatment group are irradiated with 6 J/cm^2 of simulated sunlight (+Irr) while the remaining half are held at room temperature in the dark (−Irr). After a post-exposure incubation period of 18 to 24 hours, relative viability is determined in both the irradiated (+Irr) and non-irradiated (−Irr) treatment groups by measuring the enzymatic conversion of the vital dye MTT into a blue formazan salt that is measured photometrically after extraction from the tissues. Phototoxic potential can be estimated by comparing the relative reduction in viability in each irradiated treatment group to that of the equivalent non-irradiated treatment group.
Scientific basis including MoA	The test chemical is applied topically to a three-dimensional RhE tissue, composed of human-derived epidermal keratinocytes that have been cultured to form a multilayered, highly differentiated model of the human epidermis [51]. It consists of organized basal, spinous and granular layers, and a multilayered *stratum corneum* containing intercellular lamellar lipid layers representing main lipid classes analogous to those found *in vivo*. In comparison with monolayer culture system, the organic structure (multilayered and differentiated epidermis) and the presence of barrier function (*stratum corneum*) simulate more closely the *in vivo* situation and allow topical applications of a large panel of chemicals with different physiochemical properties.

Protocol available	OECD TG498 [25, 52]
Strengths and weakness	Strengths - The RhE tissues can also tolerate UVB exposure, in comparison with monolayer culture system. - A wide variety of chemicals can be tested in RhE PT.- Can also be used to evaluate risk (e.g., NOEL/C) [23] Weakness -So far, the method has only been validated for one tissue model which might not be available in some countries.
Applicability domain and limitations	Applicability - The test method is applicable to substances, complex mixture, and formulations. Limitations - Test chemicals with potent UV absorption in the same range as MTT formazan, or test chemicals able to directly reduce the vital dye MTT may interfere with the cell viability measurements (however can be addressed using specific controls described in the TG).
Predictive capacity	An initial test method pre-validation reported in 1999 with a sensitivity of 86.7% and specificity of 93.3% (set of 10 chemicals tested twice independently in three laboratories). Assay performance of RhE PT was further supported by follow-up studies [25].
Reliability	The reliability and relevance of the in vitro RhE PT was evaluated in multiple studies [25].

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2

DEFINED APPROACHES TO TESTING AND ASSESSMENT AND THEIR ROLE WITHIN IATA FOR PHOTOTOXICITY

22. As an example, an integrated photosafety testing approach, or a decision tree, is presented in Figure 2. As described previously (*See section Introduction and Scope*), tested chemicals can be subjected to initial assessment of phototoxic potential, such as UV absorption [53] and/or ROS assay [26], for clarification of its photoreactivity. If these testing systems give 'negative' prediction in any one of the initial assessment step, further photosafety assessments would not be required. If the result is 'positive,' one continues to follow the decision tree to the next step, to perform further assessments employing 3T3 NRU PT [54] and/or RhE PT [25]. Based on characteristics of these studies described in previous section, applicability of test compound would be evaluated. Appropriate study or -ies would be identified. When both studies are applicable, 3T3 NRU PT would be prioritized for the abundance of background data. For the hazard assessment, the outcome from this step may be used for phototoxic hazard categorization of the test chemicals, and a step-wise tiered approach can be used at this step. For example, if the outcome from 3T3 NRU PT is positive, the test chemical is subjected to the RhE PT as a follow-up testing. No further testing would be needed if the chemicals exhibit no significant phototoxic effects in these testing systems in 3T3 NRU PT or RhE PT. In case where positive predictions are made at this step, further assessment on the skin and eye distribution of the test chemical may be beneficial and important for risk assessment

[55]. For example, even if test chemicals were found to be phototoxic in the *in vitro* phototoxicity testing systems, the *in vivo* phototoxic risk might not be so high as long as the chemicals did not show enough distribution and/or accumulation at the light-exposed tissues such as skin and eyes. In this context, toxicokinetic testing can be applied to the tested chemicals with "positive" prediction by 3T3 NRU PT or RhE PT. At this final step, nominal dose/intake, toxicokinetic behavior and phototoxic potential would be quite different among tested chemicals; therefore, careful consideration on experimental conditions and chemical suitability should be made in order to avoid false negative predictions.

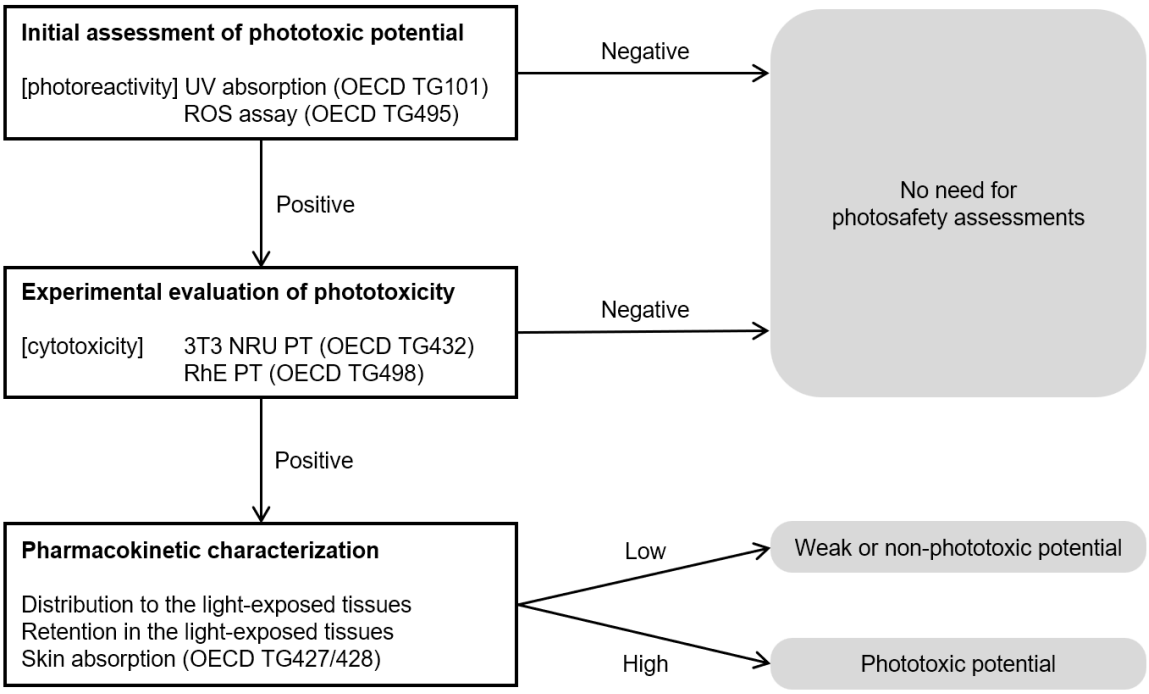

Figure 2: An example of integrated photosafety testing approach. In the 3T3 NRU PT, 'equivocal phototoxicity' prediction should be treated as positive.

23. The intent of this guidance document is to provide an overview of information sources that can be used within an IATA for phototoxicity with consideration for strength and weakness of each information source and an example of how the different information sources can be used within an IATA to increase confidence for the regulatory decision on the prediction of phototoxic or non-phototoxic potential of chemicals.

24. The case studies documented and referenced in this guidance document do not imply acceptance or endorsement by any Member Country or OECD. They are intended only to provide a perspective of how individual information sources and defined approaches, used on their own or within an IATA for phototoxicity, should be reported and to illustrate what forms these may take, whether they are statistically derived, or qualitative in nature, and intended assessment purposes (i.e. hazard versus potency prediction).

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