chemicals were unevaluable by the ROS assay because of their limited solubility, and 3 non-phototoxic chemicals (**23**, **28** and **31**) were falsely identified as phototoxic and/or photosensitive (Fig. 2C).

With respect to the aROS assay, only 2 chemicals (**13** and **26**) had to be diluted for photosafety evaluation, and *c.* 94% of tested chemicals could be successfully assessed at 200 μM (Fig. 2B). In the aROS assay, most tested chemicals exhibited a similar photochemical behavior to that observed in the ROS assay. Although reliable photosafety evaluation of glibenclamide (**8**) could not be achieved by the ROS assay owing to the limited solubility, the aROS assay provided consistent prediction results. The solubility of glibenclamide (**8**) in ROS assay buffer, containing *p*-nitrosodimethylaniline (50 μM), and imidazole (50 μM) in 20 mM NaPB (pH 7.4), was determined to be 114 μM (56.3 μg ml^{-1}), whereas that in aROS assay buffer was increased to 215 μM (106.2 μg ml^{-1}). The expansion of the applicability domain should be attributable to the solubilizing function of co-existing BSA at an equimolar concentration. Most notably, transition in photochemical properties was observed for some non-phototoxic chemicals such as aspirin (**23**), penicillin G (**28**) and warfarin Na (**31**) in the presence of BSA, and they were then correctly predicted as non-phototoxic by the aROS assay. There also appeared to be a photochemical transition in two phototoxins (**11** and **12**), and the aROS assay demonstrated that these phototoxins were falsely deduced to be less phototoxic. In the present study, the photochemical behavior of tested chemicals could vary in the presence of serum albumin, possibly affecting the prediction capacity in photosafety assessment. With respect to hexachlorophene (**12**), this agent has been reported to cause photocontact dermatitis when applied topically in humans; however, hexachlorophene (**12**) was identified to be non-phototoxic in both *in vitro* 3T3 NRU phototoxicity testing (Jones and King, 2003) and *in vivo* animal experiments (Spielmann *et al.*, 1998). Thus, *in vivo* and *in vitro* phototoxicity of hexachlorophene (**12**) is a matter of controversy, and it is still doubtful whether the reasonable photosafety assessment of hexachlorophene (**12**) can be achieved by ROS and/or an aROS assay. There is also no denying that the photosensitivity of hexachlorophene (**12**) might be attenuated through interaction with serum albumin when applied orally.

The prediction capacity of the ROS and aROS assays is summarized in Fig. 2C. The individual specificities for ROS and aROS assays were 62.5% and 100%, respectively. The positive and negative predictivities for ROS and aROS assays were found to be 87.0/100% (ROS assay) and 100/81.8% (aROS assay). Thus, unlike the ROS assay, the aROS assay provided no false positives, but two false negatives instead.

The aROS assay might be superior to the ROS assay in terms of the wide applicability domain as well, and such a high prediction capacity may indicate its potential as a reliable photosafety assessment tool. The aROS assay may be efficacious for early hazard identification in photosafety assessment; however, a thorough understanding of the assay limitations should be obtained to avoid overestimation and misleading conclusions in exploratory and regulatory use.

Spectroscopic Analysis of the Interaction between Phototoxin and Albumin

In light of the photochemical transition of some chemicals in the presence of BSA, further studies were carried out to clarify the possible relationship between molecular interaction and photochemical change in more detail. To evaluate the interaction between serum albumin and chemical, UV spectral patterns of two standards (**1** and **2**) and the tested chemicals with high photochemical transitions were measured in the presence and absence of BSA; these included diclofenac Na (**7**), hexachlorophene (**12**), lomefloxacin HCl (**15**) and warfarin Na (**31**) (Fig. 3). As radiation below 300 nm in sunlight hardly reaches the surface of the earth owing to effective absorption by the stratospheric ozone layer, the present spectroscopic study focused on spectral transition in UVA, partial UVB and VIS regions. All tested chemicals showed a strong absorption in the UVA/B range, and UV spectral patterns of some tested compounds, including sulisobenzone (**2**), diclofenac Na (**7**), hexachlorophene (**12**) and warfarin Na (**31**), were found to be different between protein-free and albuminous solutions (Fig. 3B, C, D and F). These spectral transitions would be highly indicative of protein binding with these chemicals, and the observations were partly in agreement with previous reports on potent serum protein-binding properties of these chemicals (Gulden *et al.*, 2003; Ji *et al.*, 2002). Although the aROS assay demonstrated the considerable changes in photochemical properties of quinine HCl (**1**) and lomefloxacin HCl (**15**) in the presence of serum albumin, no spectral differences were observed in these chemicals with or without serum albumin (Fig. 3A and E). Thus, there was a data discrepancy between the spectroscopic and photochemical data; therefore, an additional drug–protein interaction study was employed for further characterization.

In order to ensure interaction of chemicals with serum albumin, fluorescence quenching analysis of BSA with or without each chemical was also carried out (Fig. 4). The intrinsic fluorescence intensity of BSA measured before and after the addition of each chemical could provide information on the structural changes of BSA (Kandagal *et al.*, 2006). Theoretically, the fluorescence intensity of a chemical can be decreased by several types of molecular interaction; therefore, the quenching of fluorescence would reflect the molecular interaction. With the addition of some chemicals (**2**, **7**, **12** and **31**) at a concentration of 20 μM, the fluorescence intensity of BSA decreased by *c.* 19%, 45%, 67% and 21%, respectively. The interaction of a chemical with serum albumin can lead to changes in the dielectric environment of at least one of the two indole moieties at tryptophan residues in BSA as the chromophore may be placed in a more hydrophobic environment (Yuan *et al.*, 1998). There is also the probability that the quenching of BSA by these chemicals is as a result of energy transfer from excited-state tryptophan residues to the bound chemical. A general prerequisite for energy transfer to occur by Förster mechanisms is that the emission band of the donor overlaps the absorption band of the acceptor. Basically, the Förster mechanisms operates through the Coulombic interactions between the donor and the acceptor transition dipoles, and the resonant coupling of the donor and acceptor leads to energy transfer (Chen and Kernohan, 1967). According to the UV spectral data (Fig. 3), all tested chemicals would meet this prerequisite, whereas no fluorescence spectral transitions were observed for quinine HCl (**1**) and lomefloxacin HCl (**15**). In this study, outcomes from the fluorescence experiment were consistent with those from the UV spectral study, and, of all 6 chemicals tested here, 4 chemicals (**2**, **7**, **12**, and **31**) were found to bind with BSA under the current experimental conditions. Previously, plasma protein binding ratios of quinine HCl (**1**) and lomefloxacin

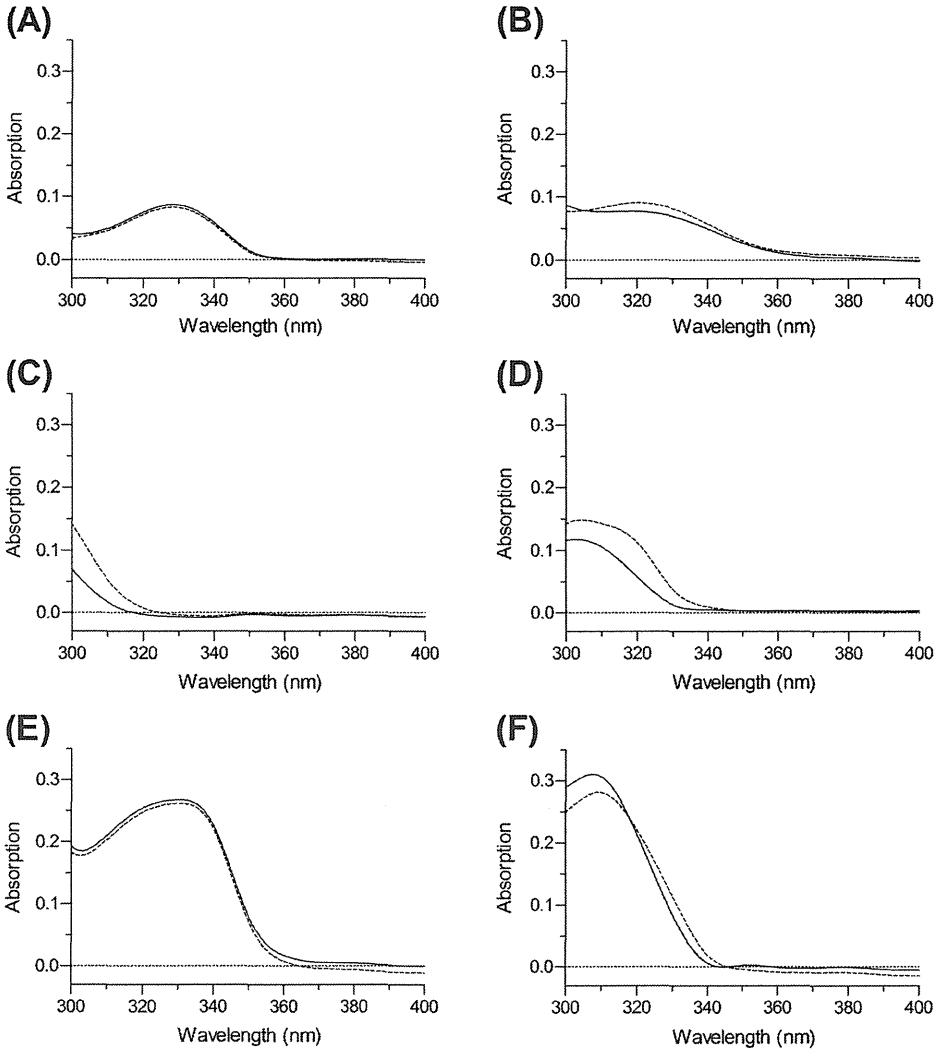

Figure 3. UV/VIS spectral patterns of tested chemicals (20 μM) with or without an equimolar concentration of bovine serum albumin (BSA) in 20 mM sodium phosphate buffer (pH7.4). (A) quinine HCl (**1**), (B) sulisobenzone (**2**), (C) diclofenac Na (**7**), (D) hexachlorophene (**12**), (E) lomefloxacin HCl (**15**) and (F) warfarin Na (**31**). Solid line, chemical alone; and dashed line, chemical with BSA.

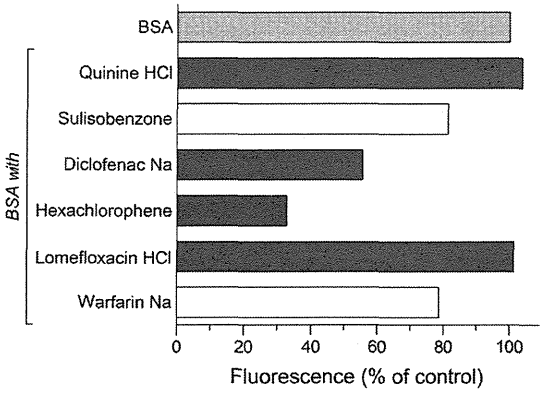

Figure 4. Fluorescence quenching analysis on bovine serum albumin (BSA) (2 μM) with or without tested chemical (2 μM). Filled bar, phototoxic chemical; and open bar, non-phototoxic chemical.

HCl (**15**) were determined to be *c.* < 70% and 10%, respectively, whereas the addition of copper ion promoted the interaction of lomefloxacin HCl (**15**) with BSA (Lu *et al.*, 2007). In contrast, 4 chemicals (**2**, **7**, **12** and **31**) exhibited much higher plasma protein binding (>95%) according to the manufacturers' reports, and these previous data would be partly supportive of the present observations. In addition to the binding amount, binding sites of the tested chemicals might also be attributable to the different outcomes from the fluorescence quenching assay.

From these findings, a molecular interaction between chemical and BSA cannot fully explain the changes in ROS generation from some chemicals in albuminous solution. However, the transition in UV spectral patterns and/or fluorescent intensity might be negligible if the chemicals interact with non-chromophore moieties and/or non-fluorescent residues in BSA. Further clarification on the BSA–drug interaction or the possible main effector might be of value to better understand assay limitations and

further modification of the ROS assay to improve the predictability of phototoxic risk and to avoid misleading data.

Conclusion

In the present study, an aROS assay system was developed using an albuminous solution for photosafety assessment under experimental biomimetic conditions. The validation study demonstrated the high robustness of the aROS assay and improved applicability to poorly soluble chemicals. Further assessments on 31 test samples were also indicative of a good predictive capacity of the aROS assay for photosafety evaluation. Comparing outcomes from the ROS assay with those from the aROS assay, the photosensitivity of some chemicals clearly varied in the presence of serum albumin, the mechanism of which might partly involve the molecular interaction. The determination of ROS in the albuminous solution might partly reflect the photochemical behavior of photosensitizers *in vivo*, and the aROS assay system would be more effective to predict the phototoxic potential of pharmaceutical substances with a wide range of applicability.

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Research Article

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Establishment and intra-/inter-laboratory validation of a standard protocol of reactive oxygen species assay for chemical photosafety evaluation

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ABSTRACT: A reactive oxygen species (ROS) assay was previously developed for photosafety evaluation of pharmaceuticals, and the present multi-center study aimed to establish and validate a standard protocol for ROS assay. In three participating laboratories, two standards and 42 coded chemicals, including 23 phototoxins and 19 nonphototoxic drugs/chemicals, were assessed by the ROS assay according to the standardized protocol. Most phototoxins tended to generate singlet oxygen and/or superoxide under UV–vis exposure, but nonphototoxic chemicals were less photoreactive. In the ROS assay on quinine (200 μM), a typical phototoxic drug, the intra- and inter-day precisions (coefficient of variation; CV) were found to be 1.5–7.4% and 1.7–9.3%, respectively. The inter-laboratory CV for quinine averaged 15.4% for singlet oxygen and 17.0% for superoxide. The ROS assay on 42 coded chemicals (200 μM) provided no false negative predictions upon previously defined criteria as compared with the *in vitro/in vivo* phototoxicity, although several false positives appeared. Outcomes from the validation study were indicative of satisfactory transferability, intra- and inter-laboratory variability, and predictive capacity of the ROS assay. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: phototoxicity; photoreactivity; reactive oxygen species; validation; inter-laboratory precision

INTRODUCTION

Phototoxic skin responses can be caused by the combined effects of UV–vis irradiation and external phototoxins (Stein and Scheinfeld, 2007), and increasing attention has been drawn to drug-induced phototoxicity because of the gradual expansion in the UV portion of the solar spectrum. For photosafety assessment in pharmaceutical development, a number of photosafety

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screening systems have been suggested over the past few years, including a UV absorption system (Henry *et al.*, 2009), a photohemolysis model (Selvaag, 1997), a cutaneous phototoxic reaction model using the human reconstituted epidermis Episkin (Portes *et al.*, 2002), a 3T3 neutral red uptake phototoxicity test (3T3 NRU PT) (Spielmann *et al.*, 1994b), a reactive oxygen species (ROS) assay (Onoue and Tsuda, 2006) and a DNA-photocleavage assay (Onoue *et al.*, 2008a). In addition to these *in vitro* prediction tools, the deductive estimation of risk from existing knowledge (DEREK), an *in silico* approach, has also been proposed on the basis of structural alerts (Barratt *et al.*, 2000), allowing quick photosafety evaluation of a new chemical, even before its chemical synthesis. These photosafety assessment tools might also be effective for clarifying the mechanism of phototoxicity for chemicals, although the results from *in vitro* photosafety testing would not always reflect clinical observations on the drug-induced phototoxicity. No *in vivo* animal phototesting has been accepted for regulatory purposes to date in the USA and EU. As regulatory agencies recommend the implementation of the 3Rs principle (refinement, reduction and replacement), the interest in development of *in vitro* methods and the use of mechanistic information has been increasing in hazard identification and characterizationof the steps of the risk assessment process (Holmes *et al.*, 2010).

The ROS assay was designed for photoreactivity assessment of pharmaceutical substances, the principle of which is monitoring of type I and II photochemical reactions in the test chemicals exposed to simulated sunlight (Onoue and Tsuda, 2006). When a drug molecule in the skin can absorb photon energy from exposed sunlight, mainly two photochemical reactions appear: (i) electron or hydrogen transfer leading to formation of free radical species (type I photochemical reaction) and (ii) energy transfer from excited triplet photosensitizer to oxygen or biomolecules (type II photochemical reaction; Onoue *et al.*, 2009). Through these photochemical reactions, the excitation of phototoxic drugs by sunlight exposure may give rise to ROS such as singlet oxygen and superoxide, and these photochemical processes can be a trigger for drug-induced phototoxicity (Brendler-Schwaab *et al.*, 2004; Epstein and Wintroub, 1985). These chemical responses of phototoxins in the very early stages of phototoxic events could be a theoretical rationale for the use of ROS assay in early qualitative photosafety assessment. In addition, the combined use of ROS data and pharmacokinetic profiles was effective for quantitative evaluation of *in vivo* phototoxic risk with high clinical relevance (Seto *et al.*, 2011). To improve the performance and reliability of the ROS assay, a simple multiwell plate-based ROS assay system was previously designed using a quartz reaction container, which would allow the reduction of sample volume, improvement of assay productivity and highly uniform irradiation (Onoue *et al.*, 2008b, 2008c). The experimental conditions of the ROS assay were then optimized with focus on irradiation uniformity, UV intensity, UV-exposure period, temperature and co-solvent systems, although intra- and inter-laboratory accuracy and precision have never been fully elucidated.

The present study was undertaken to establish and validate a standard protocol for ROS assay for exploratory and/or regulatory photosafety assessment, and the validation study was thoroughly supervised by the Japanese Center for the Validation of Alternative Methods (JaCVAM). Inter- and intra-laboratory validation studies were carried out with the aim of evaluating the transferability, the intra- and inter-laboratory variability and the predictive capacity of the ROS assay using two standard chemicals and 42 coded chemicals, including 23 phototoxins and 19 nonphototoxic drugs/chemicals.

MATERIALS AND METHODS

General Conditions of the Study

The validation study was coordinated and sponsored by JaCVAM. The validation management team (VMT) was organized under the JaCVAM and comprised the trial coordinator (Dr Hajime Kojima, JaCVAM/NIHS, Japan), the assistant trial coordinator (Dr Kazuhiro Hosoi, Santen Pharmaceutical Co. Ltd, Japan), the chemical management group (Dr Tsuguto Toda, Shionogi & Co. Ltd, Japan; Dr Yasuhiro Matsumoto, ASKA Pharmaceutical Co. Ltd, Japan; and Dr Manfred Liebsch, International Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments, ZEBET, Germany), the data analysis group (Dr Naoto Osaki, Taisho Pharmaceutical Co. Ltd, Japan), the quality assurance group (Dr Satoru Kawakami, Asahi Kasei Pharma Corporation, Japan) and representatives of participating laboratories. The roles of the VMT were to design the study, to guide and facilitate the prevalidation/validation process, to evaluate the results and on the basis of this, render subsequent decisions during the progress of the study, as well as analyzing the outcomes from the studies. The lead laboratory was responsible for directing the technical aspects of the study.

Preceding the validation study, the prevalidation study was conducted to assess transferability from the lead laboratory to participating laboratories and intra-/inter-laboratory reproducibility using 13 un-coded chemicals. The prevalidation study was undertaken for the training of the laboratory personnel, the refinement of the protocol and the data analysis sheet.

The validation study and the quality assurance were carried out in the spirit of Good Laboratory Practice (GLP), although not all the participating laboratories routinely worked under GLP certification. Namely, the participating laboratories conducted the experiments in accordance with the protocol provided by the VMT. All raw data and the data analysis sheet were pre-checked for quality in each laboratory and then reviewed by the quality assurance group of the VMT. The results accurately reflect the raw data.

Participating Laboratories

The three participating laboratories are as follows: Laboratory 1 (Lab 1), Mitsubishi Tanabe Pharma Corporation, Safety Research Laboratories; Laboratory 2 (Lab 2), Food and Drug Safety Center, Hatano Research Institute; and Laboratory 3 (Lab 3, lead laboratory), University of Shizuoka, School of Pharmaceutical Sciences.

Chemicals and reagents

Chemicals for the validation study were selected by chemical management group of the VMT in cooperation with Dr Manfred Liebsch (ZEBET), the Europe Center for the Validation of Alternative Methods (ECVAM), the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the Korean Center for the Validation of Alternative Methods (KoCVAM). According to the reported *in vitro/in vivo* photosafety information and clinical observations (Durbize *et al.*, 2003; Moore, 2002; Motley and Reynolds, 1989; Onoue *et al.*, 2010; Peters and Holzhutter, 2002; Portes *et al.*, 2002; Spielmann, 1994; Spielmann *et al.*, 1994a, 1994b, 1998a, b, 1995; Trevisi *et al.*, 1994) and in-house assay results

Table 1. Chemicals tested in the validation study

No.	Chemical name	CAS no.	Form	Photosafety information			References
				3 T3 NRU PT	Animal	Human	
Positive/negative controls							
1	Quinine HCl	6119-47-7	Solid	+	+	+	Moore (2002); Onoue *et al.* (2010)
2	Sulisobenzone	4065-45-6	Solid	−		−	Onoue *et al.* (2010); Portes *et al.* (2002)
Phototoxic drugs							
3	Acridine	260-94-6	Solid	+	+	+	Peters and Holzhutter (2002); Spielmann *et al.* (1998a)
4	Acridine HCl	17784-47-3	Solid	+	+	+	Spielmann *et al.* (1998a, 1998b)
5	Amiodarone HCl	19774-82-4	Solid	+	+	+	Onoue *et al.* (2010); Spielmann *et al.* (1995, 1998a)
6	Chlorpromazine HCl	69-09-0	Solid	+	+	+	Onoue *et al.* (2010); Spielmann *et al.* (1995, 1998a)
7	Doxycycline HCl	10592-13-9	Solid	+	+	+	Onoue *et al.* (2010); Spielmann (1994); Spielmann *et al.* (1995)
8	Fenofibrate	49562-28-9	Solid	+		+	Peters and Holzhutter (2002); Spielmann *et al.* (1998a)
9	Furosemide	54-31-9	Solid	+/−		+	Onoue *et al.* (2010); Peters and Holzhutter (2002); Spielmann *et al.* (1998a)
10	Ketoprofen	22071-15-4	Solid	+	−	+	Onoue *et al.* (2010); Spielmann *et al.* (1998a)
11	6-Methylcoumarine	92-48-8	Solid	+	+	+	Peters and Holzhutter (2002); Spielmann (1994); Spielmann *et al.* (1995)
12	8-Methoxy psoralen	298-81-7	Solid	+	+	+	Onoue *et al.* (2010); Spielmann (1994); Spielmann *et al.* (1995)
13	Nalidixic acid	389-08-2	Solid	+	+	+	Onoue *et al.* (2010); Peters and Holzhutter (2002); Spielmann *et al.* (1998a)
14	Nalidixic acid (Na salt)	3374-05-8	Solid	+	+	+	Peters and Holzhutter (2002); Spielmann *et al.* (1998a)
15	Norfloxacin	70458-96-7	Solid	+	+	+	Onoue *et al.* (2010); Peters and Holzhutter (2002); Spielmann *et al.* (1998a)
16	Ofloxacin	82419-36-1	Solid	+	+	+	Moore (2002)
17	Piroxicam	36322-90-4	Solid	−	−	+	Moore (2002); Spielmann (1994); Spielmann *et al.* (1995)
18	Promethazine HCl	58-33-3	Solid	+		+	Onoue *et al.* (2010); Spielmann *et al.* (1995, 1998a)
19	Rosiglitazone	122320-73-4	Solid	+			a)
20	Tetracycline	60-54-8	Solid	+	+	+	Spielmann (1994); Spielmann *et al.* (1994a, 1994b, 1995)
Phototoxic chemicals							
21	Anthracene	120-12-7	Solid	+	+	+	Spielmann *et al.* (1998a, 1998b)
22	Avobenzone	70356-09-1	Solid	+		−	Motley and Reynolds (1989); Trevisi *et al.* (1994)
23	Bithionol	97-18-7	Solid	+	+	+	Spielmann *et al.* (1994a, 1994b); Spielmann *et al.* (1995, 1998b)
24	Hexachlorophene	70-30-4	Solid	−	−	+	Durbize *et al.* (2003); Peters and Holzhutter (2002); Spielmann *et al.* (1998a)
25	Rose bengal	632-69-9	Solid	+	−	+	Spielmann (1994); Spielmann *et al.* (1995, 1998a)
Nonphototoxic drugs							
26	Aspirin	50-78-2	Solid	−			Onoue *et al.* (2010)
27	Benzocaine	94-09-7	Solid	−			Onoue *et al.* (2010)
28	Erythromycin	114-07-8	Solid	−			Onoue *et al.* (2010)
29	Phenytoin	57-41-0	Solid	−			Onoue *et al.* (2010)
30	Penicillin G	113-98-4	Solid	−			Spielmann (1994); Spielmann *et al.* (1995, 1998a)
Nonphototoxic chemicals							
31	Bumetrizole	3896-11-5	Solid	−			—[a]
32	Camphor sulfonic acid	3144-16-9	Solid	−			—[a]
33	Chlorhexidine	55-56-1	Solid	−		−	Peters and Holzhutter (2002); Spielmann (1994); Spielmann *et al.* (1995)
34	Cinnamic acid	140-10-3	Solid	−	−	−	Spielmann *et al.* (1995)
35	Drometrizole	2440-22-4	Solid	−			—[a]

Table 1. *(Continued)*

No.	Chemical name	CAS no.	Form	Photosafety information			References
				3 T3 NRU PT	Animal	Human	
36	L-Histidine	71-00-1	Solid	–			Spielmann (1994); Spielmann *et al.* (1995, 1998b)
37	Methylbenzylidene camphor	36861-47-9	Solid	–	–	–	Spielmann *et al.* (1998b)
38	Octrizole	3147-75-9	Solid	–			—[a]
39	Octyl methacrylate	688-84-6	Liquid	–	–	–	Spielmann *et al.* (1998b)
40	Octyl methoxycinnamate	5466-77-3	Liquid	–			—[a]
41	Octyl salicylate	118-60-5	Liquid	–	–	–	Spielmann *et al.* (1998b)
42	PABA	150-13-0	Solid	–	–		Peters and Holzhutter (2002); Spielmann (1994); Spielmann *et al.* (1995)
43	SDS	151-21-3	Solid	–			Spielmann *et al.* (1994a, 1994b, 1998b)
44	UV-571	125304-04-3	Liquid	–			—[a]

[a]*In vitro* phototoxicity was assessed by the 3 T3 NRU PT in the participating laboratories, according to the OECD 432 guideline.

from *in vitro* 3 T3 neutral red uptake phototoxicity testing (3 T3 NRU PT), two standard chemicals and 42 test chemicals, including 23 phototoxins and 19 nonphototoxic drugs/chemicals, were selected for the intra-/inter-laboratory validation study (Table 1).

Quinine (**1**), chlorpromazine HCl (**6**), fenofibrate (**8**), ketoprofen (**10**), 6-methycoumarine (**11**), nalidixic acid (**13**), norfloxacin (**15**), ofloxacin (**16**), piroxicam (**17**), promethazine HCl (**18**), anthracene (**21**), rose bengal (**25**), aspirin (**26**), benzocaine (**27**), erythromycin (**28**), phenytoin (**29**), penicillin G (**30**), cinnamic acid (**34**), L-histidine (**36**), 2-(2 *H*-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)phenol (octrizole, **38**), octyl methacrylate (**39**), *p*-aminobenzoic acid (PABA, **42**), sodium dodecyl sulfate (SDS, **43**), $NaH_2PO_4 2H_2O$, $Na_2HPO_4 12H_2O$, dimethyl sulfoxide (DMSO), *p*-nitrosodimethylaniline, imidazole and nitroblue tetrazolium (NBT) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Doxycycline HCl (**7**) and nalidixic acid (Na salt) (**14**) were bought from MP Biomedicals (Irvine, CA, USA). Rosiglitazone (**19**) and methylbenzylidene camphor (**37**) were purchased from Enzo Life Sciences International (Farmingdale, NY, USA) and Alfa Aesar (Ward Hill, MA, USA), respectively. Sulisobenzone (**2**), acridine HCl (**4**), furosemide (**9**), 8-methoxy psoralen (**12**), avobenzone (**22**), hexachlorophene (**24**), octyl methoxycinnamate (**40**) and octyl salicylate (**41**) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Acridine (**3**), amiodarone HCl (**5**), 6-methycoumarine (**11**), tetracycline (**20**), bithionol (**23**), 2-*tert*-butyl-6-(5-chloro-2 *H*-benzotriazol-2-yl)-4-methylphenol (bumetrizole, **31**), camphor sulfonic acid (**32**), chlorhexidine (**33**), 2-(2-hydroxy-5-methylphenyl) benzotriazole (drometrizole, **35**), and 2-(2 *H*-benzotriazol-2-yl)-6-dodecyl-4-methylphenol (UV-571, **44**) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). A quartz reaction container for high-throughput ROS assay (Onoue *et al.*, 2008b) was constructed by Ozawa Science (Aichi, Japan).

Quinine (**1**), a positive control, and sulisobenzone (**2**), a negative control, were uncoded and supplied by the VMT to participating laboratories. The remaining 42 test chemicals were coded by the VMT and supplied with essential information (physical state, weight or volume of the chemicals, specific density for liquids, storage instructions, molecular weight and conversion factor). Safety information for the test chemicals was also provided to an appropriate individual within the organization who was not involved in the study, and could be opened only in the case of an emergency. In all cases, the safety information was not opened by study directors up to the end of all experiments of the validation study. The identity of the coded chemicals was made known to the study directors at the VMT meeting after all the experiments had been completed and all experimental data were submitted to the VMT.

ROS Assay Protocol

Apparatuses

Two types of solar simulators were employed for the present validation study. In Labs 1–3, the ROS assay was conducted with use of Atlas Suntest CPS series (CPS plus in Labs 1 and 3, and CPS in Lab 2; Atlas Material Technology LLC, Chicago, IL, USA) equipped with a xenon arc lamp (1500 W). A UV special filter was installed to adapt the spectrum of the artificial light source to that of natural daylight, and the Atlas Suntest CPS series had a high irradiance capability that met CIE85/1989 daylight simulation requirements (Fig. 1). The irradiation test was carried out at 25 °C with an irradiance of ca. 2.0 mW cm^{-2} as

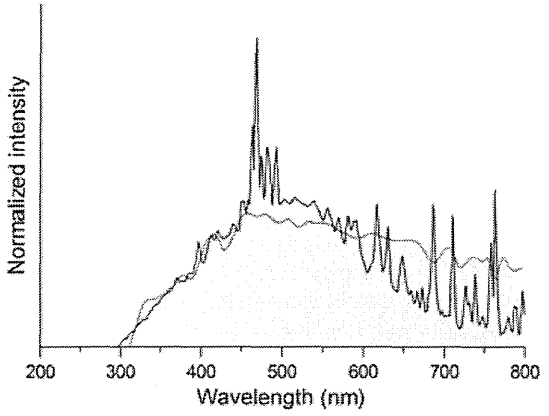

Figure 1. Spectral patterns of simulated sunlight in Atlas Suntest CPS series and standard daylight (CIE85/1989). Black line, simulated sunlight emitted from a xenon arc lamp in Atlas Suntest CPS series; and red line, standard daylight (CIE85/1989).

determined using the calibrated UVA detector Dr Hönle 0037 (München, Germany) delivered from the VMT. A quartz reaction container for high-throughput ROS assay (Onoue *et al.*, 2008b) was constructed by Ozawa Science (Aichi, Japan) and supplied by the VMT.

Preparation of test chemicals and controls

Previously, the effects of co-existing solvents on ROS assay were investigated (Onoue *et al.*, 2008b). Although some organic solvents, such as methanol, ethanol and acetonitrile, exhibited attenuation of ROS generation from UV-exposed phototoxins, addition of DMSO in ROS assay mixture resulted in slight enhancement of photochemical reactions. From these observations, taken together with the dissolving ability, DMSO was considered to be a suitable solvent to prepare stock solutions for the ROS assay. Stock solutions of the controls were prepared at 10 mM in DMSO, divided into some tubes, and stored in a freezer for up to 1 month. According to the chromatographic analysis, these stock solutions were stable for at least 1 month under current storage condition. The stock solution was thawed just before the experiment and used within the day. The coded chemicals were dissolved in DMSO at concentrations of 0.1, 1, or 10 mM just before use under UV-cut illumination or shade. All preparations were protected from light.

ROS assay procedure

The ROS assay was designed to detect both singlet oxygen and superoxide generated from photo-irradiated chemicals (Onoue *et al.*, 2008b, d). Briefly, singlet oxygen was measured in an aqueous solution by spectrophotometrically monitoring the bleaching of *p*-nitrosodimethylaniline at 440 nm using imidazole as a selective acceptor of singlet oxygen. Samples containing the tested chemical (2–200 μM), *p*-nitrosodimethylaniline (50 μM) and imidazole (50 μM) in 20 mM sodium phosphate buffer (NaPB, pH 7.4) were mixed in a tube. Two hundred microliters of the sample were transferred in a well of a plastic 96-well plate (clear, nontreat flat-bottom) and checked for precipitation under a microscope (×100) before light exposure. The plate was subjected to measurement of absorbance at 440 nm using a microplate spectrophotometer. The plate was fixed in the quartz reaction container with a quartz cover, and then irradiated with simulated sunlight for 1 h. After agitation on plate shaker, UV absorbance at 440 nm was measured. For the determination of superoxide, samples containing the tested chemical (2–200 μM) and NBT (50 μM) in 20 mM NaPB were irradiated with the simulated sunlight for 1 h, and the reduction in NBT was measured by the increase in absorbance at 560 nm in the same manner as the singlet oxygen determination. Experiments were performed in triplicate wells in three independent runs. As the final concentrations, 200 μM of test chemical solutions were subjected to the ROS assay. However, when precipitation could be observed at 200 μM under the optical microscope, additional experiments were performed at 20 μM. Further experiments should be performed at 2 μM when precipitation was still observed at 20 μM. When precipitation was observed at 2 μM in the reaction mixture, further experiment was no longer needed.

Criteria for data acceptance and judgment

The criteria for acceptability of valid assay were: (i) no precipitation of the test chemical in the reaction mixture before light exposure: (ii) no data lacking positive control, negative control, blank and chemical; and (iii) net absorbance of 0.02–1.5 in the controls and the chemical.

According to the results (mean of triplicate determinations) of the ROS assay, the photoreactivity on each test chemical should be judged to be: (i) positive with singlet oxygen ($\Delta A_{440\ nm} \times 10^3$) 25 or more and/or superoxide ($\Delta A_{560\ nm} \times 10^3$) 20 or more; or (ii) negative with singlet oxygen less than 25 ($\Delta A_{440\ nm} \times 10^3$), and superoxide ($\Delta A_{560\ nm} \times 10^3$): less than 20. From mean values of three independent experiments, a final decision should be made as to: (i) positive, above threshold level for singlet oxygen or superoxide; or (ii) negative, below threshold level for both singlet oxygen and superoxide.

RESULTS AND DISCUSSION

Transferability

Intra-laboratory reproducibility for two standard chemicals

For the exploratory and/or regulatory use of ROS assay, a standardized protocol should be transferable with high assay reproducibility. To assess the transferability of the ROS assay, ROS assay was carried out for quinine (**1**), a typical phototoxin (Moore, 2002), and sulisobenzone (**2**), a nonphototoxic chemical (Portes *et al.*, 2002), in the three laboratories participating in the validation study (Lab 1, Mitsubishi Tanabe Pharma Corporation; Lab 2, Hatano Research Institute; and Lab 3, University of Shizuoka). In all the laboratories, similar solar simulators (Atlas Suntest CPS plus for Labs 1 and 3, and Atlas Suntest CPS for Lab 2) equipped with the same light source (Xe lamp, Atlas 56-0017-94) were employed for the ROS assay. As observed previously (Onoue and Tsuda, 2006), in all the laboratories, quinine (**1**: 200 μM) had the ability to generate both singlet oxygen and superoxide under UV–vis exposure (ca. 2.0 mW cm^{-2}) for 1 h, whereas ROS generation from irradiated sulisobenzone (**2**: 200 μM) was negligible (Table 2). Without any exposure to the simulated sunlight, neither tested chemical exhibited any ROS generation, even though most experimental procedures were carried out under a normal white fluorescent lamp (data not shown). The intra-day precision of the ROS assay was evaluated by analyzing nine samples of quinine (**1**) or sulisobenzone (**2**) solutions at 200 μM. The intra-day coefficients of variation (CV) for the detection

Table 2. Intra- and inter-laboratory precision of ROS assay

	Generation of reactive oxygen species, Mean ± SD (CV, %)					
	Singlet oxygen (decrease in $A_{440\ nm} \times 10^3$)			Superoxide (increase in $A_{560\ nm} \times 10^3$)		
	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3
Intra-laboratory (n = 9)						
Intra-day						
Quinine	366 ± 8 (2.1)	429 ± 7 (1.5)	531 ± 10 (1.8)	306 ± 23 (7.4)	291 ± 16 (5.3)	403 ± 10 (2.6)
Sulisobenzone	−2.2 ± 1.9	0.9 ± 3.0	4.6 ± 6.3	−5.3 ± 0.5	−15.4 ± 1.2	−16.6 ± 3.2
Inter-day						
Quinine	367 ± 12 (3.3)	429 ± 7 (1.7)	534 ± 19 (3.5)	310 ± 21 (6.9)	278 ± 26 (9.3)	399 ± 10 (2.5)
Sulisobenzone	−1.7 ± 1.8	0.8 ± 3.1	−1.2 ± 2.8	−6.2 ± 0.9	−12.7 ± 3.6	−12.1 ± 2.1
Inter-laboratory (n = 9)						
Quinine		442 ± 68 (15.4)			330 ± 56 (17.0)	
Sulisobenzone		1.4 ± 4.3			−11.7 ± 4.8	

Quinine (**1**) or sulisobenzone (**2**) was dissolved in 20 mM sodium phosphate buffer (pH 7.4) at a final concentration of 200 μM and exposed to simulated sunlight (ca. 2.0 mW cm^{-2}) for 1 h. Data represent mean ± SD of three repeated experiments for intra-day ($n = 9$) precision and three repeated experiments for inter-day precision (day 1, 2, and 3; $n = 9$). Values in parentheses are coefficients of variation (%).

of singlet oxygen and superoxide generated from irradiated quinine (**1**) were found to range from 1.5 to 2.1%, and from 2.6 to 7.4%, respectively. In addition, the inter-day CV values for quinine (**1**) in Labs 1–3 varied from 1.7 to 3.5% for singlet oxygen, and from 2.5 to 9.3% for superoxide. On the basis of these data, determination of the singlet oxygen tended to exceed that of superoxide in its precision. In all laboratories, the CV obtained was below 10%, suggesting good intra- and inter-day precision, and thus could be considered reproducible across experiments and across laboratories.

Inter-laboratory reproducibility for two standard chemicals

For the inter-laboratory reproducibility assessment, the same assay protocol and experimental conditions such as buffer system, UV irradiance and reaction container were maintained that had been used for intra-laboratory reproducibility assessment. In all the laboratories (Labs 1–3), sulisobenzone (**2**) was found to be photochemically inactive, and significant ROS generation was observed in the positive control quinine (**1**: 200 μM) with CV values of 15.4% (singlet oxygen) and 17.0% (superoxide). Thus, the successful transferability of the standardized ROS assay was also demonstrated by the satisfactory results for the inter-laboratory reproducibility. Upon these results, the VMT therefore agreed that these participating laboratories (Labs 1–3) were ready to proceed to the further inter-laboratory reproducibility study.

Predictive capacity

In addition to quinine (**1**) and sulisobenzone (**2**), the photochemical reactivities of 42 coded chemicals, consisting of 23 known phototoxins (**3–25**) and 19 nonphototoxic drugs/chemicals (**26–44**), were also assessed by the ROS assay in Labs 1–3, with the aim of evaluating its predictive capacity and inter-laboratory reproducibility in more detail (Table 3). Of all tested chemicals, assessment on 25–28 chemicals (60–67% of total) could be made at the concentration of 200 μM, and 14–17 chemicals had to be diluted to a final concentration of 2 or 20 μM because of limited solubility in aqueous media. In particular, assay of amiodarone HCl (**5**), anthracene (**21**), avobenzone (**22**), octrizole (**38**) and UV-571 (**44**) was available only at 2 μM in most laboratories. Rose bengal (**25**) exhibited intense UV–vis absorption with a molar extinction coefficient of 90 400 M^{-1} cm^{-1} at 559 nm (Seybold *et al.*, 1969), and the absorption spectrum of rose bengal overlapped with that of nitroblue diformazan, a specific indicator for superoxide used in the ROS assay. Herein, for rose bengal (**25**), only singlet oxygen could be measured owing to the severe spectral interference at 560 nm. In the three participating laboratories, all phototoxins at concentrations of 20 and 200 μM demonstrated potent ability to generate singlet oxygen, superoxide, or both under UV–vis exposure; however, generation of ROS was negligible for some phototoxins at 2 μM. With respect to the nonphototoxic drugs/chemicals, similar photochemical reactivity was also seen among the tested substances, although some exhibited potent photoreactivity in a few laboratories.

Previously, ROS assay was carried out for 39 marketed drugs and chemicals (200 μM) including 33 phototoxins and six nonphototoxic compounds (Onoue *et al.*, 2008b), and there appeared be clear differences between phototoxins and nonphototoxic compounds in their ROS generation ability under light exposure. According to 2D-plot analysis on the obtained ROS data, tentative classification criteria [25 for singlet oxygen ($\Delta A_{440\ nm} \times 10^3$) and 20 for superoxide ($\Delta A_{560\ nm} \times 10^3$)] were proposed to discriminate the phototoxins from nonphototoxic substances. These criteria were defined for the ROS assay on tested chemicals at a concentration of 200 μM, and, therefore, they would not be theoretically applicable to the outcomes from assays at a lower concentration (2 or 20 μM). However, the tested chemicals could be identified to be phototoxic as long as the ROS data at 2 or 20 μM surpassed these tentative classification criteria, so that these criteria might still be available for positive prediction on the diluted samples. In contrast, it would be challenging to make negative predictions on the basis of ROS assay under dilution. For further comparison, the results from the ROS assay were analyzed on a 2D plot of singlet oxygen vs superoxide generation for various substances at different concentrations

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Table 3. Outcomes from reactive oxygen species (ROS) assay in three laboratories (Labs 1–3)

No.	Chemical name	Lab 1						Lab 2						Lab 3					
		Singlet oxygen[a]			Superoxide[b]			Singlet oxygen[a]			Superoxide[b]			Singlet oxygen[a]			Superoxide[b]		
		1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
Phototoxic drugs																			
3	Acridine	223	221	223	192	172	168	229	218	214	191	209	190	225	233	222	228	231	215
4	Acridine HCl	215	215	218	176	181	164	226	216	224	198	212	181	224	214	223	211	215	215
5	Amiodarone HCl	**36**	**51**	**48**	**0**	**2**	**0**	**25**	**38**	**28**	**3**	**5**	**−5**	**33**	**9**	**22**	**−16**	**−13**	**−5**
6	Chlorpromazine HCl	−22	−32	−23	87	84	78	−2	−13	−6	97	99	109	−18	−41	−15	84	106	97
7	Doxycycline HCl	166	160	160	247	249	261	222	234	222	413	437	411	271	269	262	353	486	342
8	Fenofibrate	*161*	*161*	*161*	*−9*	*−12*	*−12*	**5**	**18**	**11**	**−6**	**17**	**−9**	*124*	*202*	*173*	*0*	*−39*	*−31*
9	Furosemide	145	146	144	52	50	54	138	131	133	62	67	67	227	238	224	115	121	102
10	Ketoprofen	224	220	206	80	88	87	245	259	240	107	117	109	358	362	368	130	122	137
11	6 – Methylcoumarine	106	96	99	62	70	67	103	122	142	87	110	100	114	111	120	109	128	130
12	8 – Methoxy psoralen	65	77	70	23	30	31	81	79	60	87	103	92	83	101	78	76	138	113
13	Nalidixic acid	134	130	119	294	356	314	147	144	145	254	206	252	348	185	182	355	271	264
14	Nalidixic acid (Na salt)	125	124	119	299	341	320	144	140	149	218	192	211	183	165	183	287	233	203
15	Norfloxacin	164	171	169	149	149	139	188	222	200	132	120	119	215	219	214	145	139	113
16	Ofloxacin	126	125	117	420	439	446	132	137	149	288	292	292	193	203	192	351	228	274
17	Piroxicam	168	160	153	*4*	*3*	*7*	207	230	221	*56*	*47*	*43*	191	210	232	*28*	*38*	*26*
18	Promethazine HCl	43	46	39	35	33	36	91	89	103	43	51	48	70	62	84	67	86	86
19	Rosiglitazone	57	54	54	*15*	*17*	*15*	90	87	93	*27*	*29*	*35*	118	104	131	*41*	*36*	*29*
20	Tetracycline	134	129	129	146	101	123	167	160	166	240	255	239	200	194	200	197	216	218
Phototoxic chemicals																			
21	Anthracene	**5**	**3**	**4**	**4**	**4**	**4**	**−2**	**7**	**6**	**3**	**8**	**−3**	*261*	*340*	*272*	**39**	**48**	**1**
22	Avobenzone	*58*	*60*	*65*	*13*	*9*	*18*	**0**	**12**	**10**	**29**	**36**	**25**	*142*	*121*	*117*	**52**	**19**	**32**
23	Bithionol	81	68	72	34	30	33	137	143	140	**13**	**15**	**15**	81	113	114	*21*	*28*	*24*
24	Hexachlorophene	227	226	217	10	8	6	246	255	258	**−6**	**15**	**−3**	318	333	355	6	22	7
25	Rose bengal	608	589	607	–	–	–	631	634	667	–	–	–	682	685	679	–	–	–
Nonphototoxic drugs																			
26	Aspirin	−2	3	1	−1	−1	0	1	−1	1	0	−6	−4	1	2	4	−12	−15	10
27	Benzocaine	0	4	3	0	1	0	9	1	−12	−4	12	6	6	3	−2	9	20	0
28	Erythromycin	0	−4	−3	1	4	2	6	−1	13	14	4	35	−16	5	8	4	8	6
29	Phenytoin	2	6	4	35	17	26	0	−4	2	52	53	48	1	19	3	63	55	32
30	Penicillin G	0	5	4	4	11	16	0	−1	4	37	36	34	17	6	9	11	26	40
Nonphototoxic chemicals																			
31	Bumetrizole	*−6*	*−16*	*−19*	*−2*	*2*	*9*	**−5**	**10**	**0**	**−7**	**0**	**−7**	*−7*	*−11*	*−11*	**−8**	**9**	**−6**
32	Camphor sulfonic acid	3	1	4	−4	−1	0	−1	3	2	−4	5	−2	7	−2	8	−4	−12	−22
33	Chlorhexidine	23	22	22	13	7	10	11	−9	8	13	21	16	−5	8	−12	28	35	6
34	Cinnamic acid	0	−1	2	9	10	7	5	0	−8	52	36	37	6	0	0	61	70	36
35	Drometrizole	**8**	**2**	**4**	*6*	*7*	*7*	*2*	*6*	*8*	*8*	*3*	*2*	*7*	*−4*	*−7*	*−11*	*13*	*−8*
36	L – Histidine	4	3	4	51	48	48	4	9	−3	55	−4	68	12	15	7	61	73	51

Table 3. *(Continued)*

No.	Chemical name	Lab 1						Lab 2						Lab 3					
		Singlet oxygen[a]			Superoxide[b]			Singlet oxygen[a]			Superoxide[b]			Singlet oxygen[a]			Superoxide[b]		
		1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
37	Methylbenzylidene camphor	*−4*	*−3*	*−8*	*−2*	*−3*	*−1*	*−3*	*−1*	*−1*	*4*	*7*	*−5*	*−7*	*−3*	*−4*	*−6*	*5*	*−4*
38	Octrizole	*−13*	*−11*	*−17*	*4*	*6*	*11*	**−3**	**3**	**−3**	**2**	**20**	**6**	**4**	**−6**	**1**	**56**	**47**	**22**
39	Octyl methacrylate	*10*	*4*	*6*	*0*	*0*	*−1*	*1*	*−1*	*4*	*−6*	*−1*	*−7*	*17*	*26*	*52*	*−3*	*−25*	*−38*
40	Octyl methoxycinnamate	*3*	*2*	*4*	*−5*	*−5*	*−4*	**−4**	**6**	**2**	**−5**	**2**	**1**	*6*	*2*	*5*	*−17*	*−15*	*−11*
41	Octyl salicylate	*−3*	*−3*	*−3*	*0*	*0*	*0*	*1*	*7*	*7*	*7*	*2*	*−4*	*0*	*14*	*2*	*12*	*2*	*1*
42	PABA	*−5*	*−3*	*3*	*−1*	*0*	*−1*	*2*	*2*	*7*	*−9*	*−2*	*−1*	*18*	*−8*	*5*	*10*	*−5*	*8*
43	SDS	*5*	*5*	*8*	*5*	*6*	*14*	*5*	*8*	*12*	**1**	**−4**	**1**	*18*	*17*	*15*	*16*	*5*	*3*
44	UV − 571	**−5**	**−4**	**−4**	**1**	**2**	**1**	**−10**	**7**	**−4**	**2**	**8**	**0**	*−16*	*−19*	*−15*	**10**	**4**	**15**

[a]Decrease in $A_{440\ nm} \times 10^3$, and [b] increase in $A_{560\ nm} \times 10^3$. ROS assay was basically carried out for test chemicals at a concentration of 200 μM, and ROS data on the diluted chemicals are emphasized in bold (2 μM) and italic (20 μM). Each value represents the mean of three determinations.

(Fig. 2A–C). It should be noted that most phototoxic compounds even at concentrations of 2 and 20 μM showed a significant amount of ROS generation that exceeded the threshold level. Upon the proposed criteria, most phototoxins tested here were correctly identified as phototoxic in Labs 1–3, whereas only fenofibrate (**8**) and anthracene (**21**) were unevaluable owing to the poor solubility in Lab 1 and/or 2. In addition, of all 19 nonphototoxic samples, 11–17 compounds (ca. 58–89% of total negatives) were lying in the subthreshold region. According to the defined classification criteria, four to nine compounds (**26**, **27**, **28**, **30**, **32**, **33**, **34**, **42** and **43**) were found to be nonphototoxic on the basis of ROS data at 200 μM, and the negativity for other chemicals (**31**, **35**, **37**, **40**, **41** and **44**) could not be fully proven as they were assessed at 2 or 20 μM.

Overall, reproducible prediction could be achieved among participating laboratories, in which consistency for evaluable phototoxins and nonphototoxic chemicals was found to be 100 and 70%, respectively. There appeared to be inconsistent predictions in penicillin G (**30**), chlorhexidine (**33**) and cinnamic acid (**34**), and the 2D diagram (Fig. 2A–C) demonstrated that these chemicals were plotted around the boundary. Because of the lowered classification criteria with the aim of preventing false negative prediction, the slight variation in the ROS assay might lead to discrepant phototoxicity prediction on such a chemical. The prediction capacity of the ROS assay is summarized in Fig. 2(D). The individual specificities for each laboratory were 81.8, 60 and 41.7% for Labs 1–3, respectively. The positive and negative predictivities for each laboratory were found to be 91.7/100% (Lab 1), 84.0/100% (Lab 2) and 75.9/100% (Lab 3). Thus, most notably, the provisional classification criteria based on the ROS assay at 200 μM provided no false negatives as compared with the *in vitro/in vivo* phototoxicity. Such a high negative predictivity could provide a reliable photosafety assessment, and it would be particularly valuable as first screening to identify the phototoxic potential of drug candidates.

Assay limitations

False positive predictions

In theory, the ROS assay would capture all the photochemically active substances as it could detect type I and/or II photochemical reactions induced by irradiated compounds. These photochemical reactions were observed at a very early stage of drug-induced phototoxic cascades, so that the ROS assay has been thought to be effective for photosafety evaluation of pharmaceuticals. However, there is the probability that, as well as the phototoxins, some photolabile substances would also be recognized as phototoxic by the ROS assay, because of significant ROS generation during the photodegradation processes. Namely, the ROS assay sometimes provides false positive predictions, as observed in the present validation study. Interestingly, some false positives observed here, such as phenytoin (**29**), penicillin G (**30**), chlorhexidine (**33**), cinnamic acid (**34**), L-histidine (**36**) and octyl methacrylate (**39**), were previously reported to be photodegradable and photoreactive, the mechanisms of which included radical reactions and/or electron transfer (Huvaere and Skibsted, 2009; Marin *et al.*, 2007; Puglia *et al.*, 2012; Ray *et al.*, 1996; Yuan *et al.*, 2009). This could explain in part the data discrepancy observed between the ROS assay and *in vitro/in vivo*

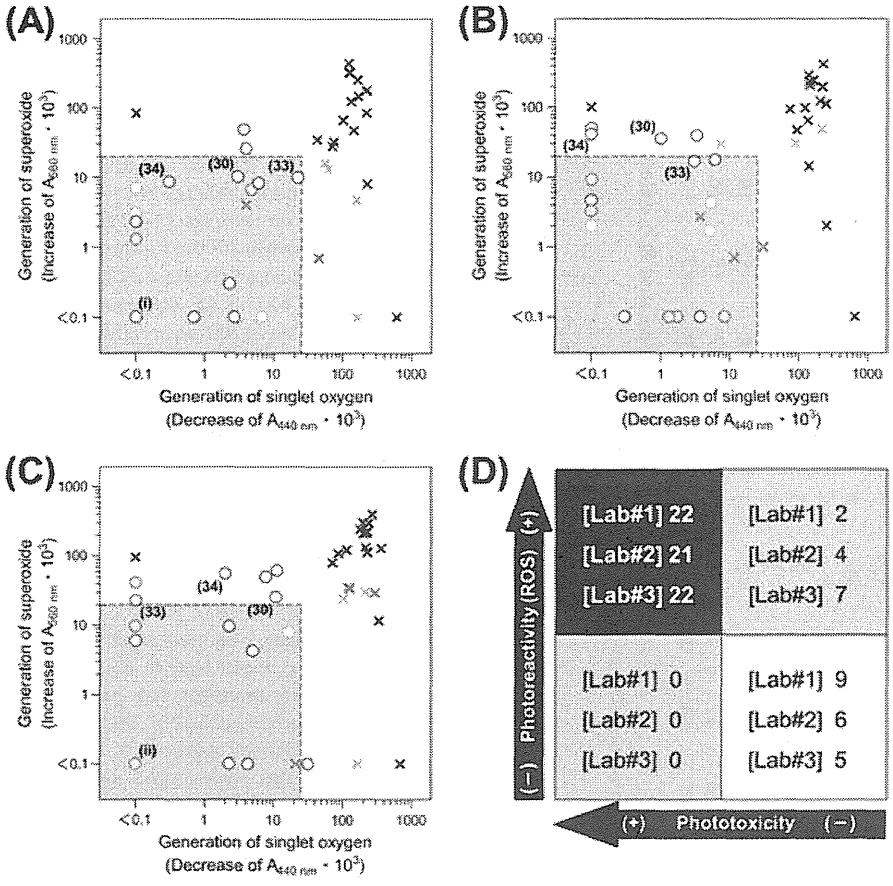

Figure 2. Predictive capacity of ROS assay in three participating laboratories. Plot of singlet oxygen data vs superoxide data for all coded test samples obtained from Lab 1 (A), Lab 2 (B), and Lab 3 (C). (○) Nonphototoxic drugs/chemicals; and (×) phototoxins at concentrations of 2 μM (red symbols), 20 μM (orange symbols) and 200 μM (black symbols). Symbols with a number represent the ROS data on penicillin G (**30**), chlorhexidine (**33**) and cinnamic acid (**34**). Each value represents mean of three experiments. According to the criteria for phototoxins (200 μM) defined previously, the shaded region is indicative of low phototoxic potential. (i) Overlapped symbols for nonphototoxic compounds: two chemicals at 20 μM and a chemical at 200 μM; and (ii) a chemical at 2 μM and two chemicals at 20 μM. (D) Positive and negative predictivity of the ROS assay as compared with the *in vitro/in vivo* phototoxicity.

phototoxicity, and better understanding of the limitation would be of great help for avoiding overestimation and misleading conclusions.

Solubility issues and potential solutions

In recent years, as many as ca. 70% of new drug candidates have shown poor aqueous solubility, and ca. 40% of marketed drugs for oral use are identified as practically insoluble in aqueous media (solubility in water $<100\,\mu g\,ml^{-1}$; Takagi *et al.*, 2006). As observed in the present validation study, there are experimental problems arising from the limited solubility of some new drug candidates in the photosafety evaluation. In such cases, appropriate options to modify the ROS assay system would be required for reliable photosafety assessment on the poorly water-soluble chemicals. It might be of some value to run the ROS assay at much lower concentration with the use of new criteria established for modified assay system. In addition, previous investigation demonstrated that the use of micellar solution systems could be effective for the photosafety assessment on poorly soluble chemicals because of the intense solubilizing potency and production of the biomembrane-mimetic environment (Onoue *et al.*, 2008d). These viable options, as well as other solubilizing approaches, would enable ROS assay for poorly water-soluble chemicals, whereas assay sensitivity and specificity should be carefully elucidated before exploratory and/or regulatory use. In particular, careful consideration and decisions should be made in order not to provide false negative predictions on photoreactivity of the tested chemicals.

CONCLUSION

In the present validation study, on the basis of the standardized protocol, photochemical reactivities of 42 coded chemicals and two standard controls were assessed by the ROS assay. Outcomes from two standard controls demonstrated the satisfactory transferability of the method and fine intra-/inter-laboratory reproducibility. Further assessments on 42 blinded test samples were also indicative of the intra-/inter-laboratory reproducibility and the predictive capacity of the ROS assay for photosafety evaluation. Thus, results from the present validation study provided sufficient support for the ROS assay as an alternative method for photosafety assessment.

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Exploratory and Regulatory Assessments on Photosafety of New Drug Entities

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Abstract: Drug-induced phototoxicity is elicited after exposure of the skin and/or eyes to topically or systemically administered pharmaceutical substances, followed by exposure to sunlight. This undesirable side effect is one of the impediments in drug discovery and development, and substantial efforts have been made to avoid drug-induced phototoxic reactions. To evaluate the phototoxic potential of compounds, effective methodologies have been developed over the past few years, and screening strategies have also been proposed for predicting *in vivo* phototoxic reactions. European and American regulatory agencies have published guidelines for predicting and avoiding drug-induced phototoxicity in an early phase of drug discovery. The guidelines have indicated the requirements for assessing the photosafety of chemicals on the basis of their photochemical behaviors and have recommended some phototoxic assessment tools for aiding new drug development. A number of phototoxic screening systems have also been proposed on the basis of the pathogenesis of drug-induced phototoxicity, and some of them have already been applied to the phototoxic evaluation of new drug entities in drug discovery and development. The present review aims to summarize the current status of research tools, screening strategy and regulations for evaluating the photosafety of new drug candidates and to introduce our thoughts on the phototoxic risk assessments of compounds.

Keywords: Hazard identification, photoallergy, photogenotoxicity, phototoxicity, photosafety evaluation.

1. INTRODUCTION

Phototoxicity is an undesirable response in the skin and eyes, triggered by exposure to sunlight, especially ultraviolet (UV) A/B radiation (UVA: 320–400 nm and UVB: 290–320 nm) and visible (VIS) light (400–700 nm). There are two types of photosensitive disorders, endogenous and exogenous phototoxicity, and the observable changes manifest after exposure to sunlight as the appearances of the following features of exaggerated sunburn such as erythema, papules, bullae and desquamation in the skin [1], and keratitis and opacification in the eyes [2]. 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, drug-induced phototoxicity has been recognized as a severe adverse effect of pharmaceuticals, the mechanism of which is exposure of the skin and/or eyes to topically or systemically administered pharmaceuticals and sunlight [3]. Several classes of pharmaceutics at clinical doses often cause phototoxic reactions in the skin and eyes [4], and perceptible adverse effects would lead to a reduction in medication compliance. In addition to the clinical issues, drug-induced phototoxicity has also thwarted development of new drug entities, and the pharmaceutical industry and regulatory agencies have struggled to predict and/or avoid the phototoxic liability.

A number of effective methodologies for evaluating the phototoxic risk of chemicals have been developed over the past few years, and guidance on the photosafety testing of medicinal products, including phototoxicity (photoirritation),

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photogenotoxicity, photoallergy and photocarcinogenicity, was established by the regulatory agencies in the US and EU in the early 2000s [5-7]. These guidelines have described tested compounds on the basis of light absorption properties, administration routes and distribution behaviors, and they have recommended some research tools for photosafety assessments in pharmaceutical research and development. In addition to the phototoxic risk assessment tools, phototoxic screening strategies have also been proposed for photosafety assessments. In the present review, we summarize the research tools, screening strategies and guidelines for photosafety evaluation of new drug candidates.

2. DRUG-INDUCED PHOTOTOXICITY

Drug-induced phototoxicity is caused by interaction of photoactivated pharmaceuticals with biomolecules, such as lipids, proteins and DNA, in the skin and eyes. Several classes of pharmaceuticals induce these types of phototoxic events [4], such as non-steroidal anti-inflammatory drugs (NSAIDs) [8, 9], antibiotics [10, 11] and diuretic agents [12]. Possible pathogenesis of drug-induced phototoxicity is described in Fig. (**1**). In the primary event, chemicals are excited by the absorption of photon energy from UVA/B radiation and VIS light. Generally, the photoactivated chemicals could release the absorbed photon energy as fluorescence, phosphorescence or heat. When the photoactivated chemicals are in close contact with other molecules, they can directly and/or indirectly react with biomolecules *via* energy transfer (type II photochemical reaction) and free radical generation (type I photochemical reaction), resulting in oxidation of biomolecules and/or photobinding to biomolecules. In particular, the photoactivated chemicals generate reactive oxygen species (ROS), including singlet oxygen ($^1O_2$) and superoxide (O_2^-) *via* type II and type I photochemical reactions, respectively, and the ROS play roles as principal intermediate species in indirect phototoxic pathways [13, 14]. On the basis of the photobiochemical reactions and clinical outcomes, drug-induced phototoxic skin responses are categorized into at least three types, photoirritation through oxidative damage on cellular lipids and proteins, photogenotoxicity through DNA damage and photoallergy through formation of photoantigens [13, 15].

2.1. Photoirritation (Narrowly Defined Phototoxicity)

Photoirritation is an inflammatory event in the skin and eyes like severe sunburn, and the event is sometimes induced by oxidative stress in the cellular membrane. The oxidative stress is triggered by both excessive accumulation of photosensitizers in the skin and eyes and exposure of the skin and eyes to a particular wavelength of light. Predominant causative reactions on photoirritation have been reported, such as photo-oxidation of lipids and proteins and photobinding of photosensitizers to some amino acid moieties in cellular membrane [16, 17].

2.2. Photogenotoxicity

Photogenotoxicity is one of the gene abnormalities caused by phototoxins, the pathogeneses of which have been categorized to be direct and indirect pathways [18, 19], and then the photogenotoxic reactions could lead to carcinogenicity. In the direct mechanisms of DNA damage by phototoxic compounds, photoexcited chemicals react with DNA *via* covalent binding, energy transfer and electron transfer, resulting in the formation of DNA photoadducts, pyrimidine dimers and oxidative DNA modifications. In the indirect pathway of DNA damage by phototoxic compounds, photoexcited chemicals yield ROS, such as singlet oxygen and superoxide, and reactive decomposition products, and then the intermediates cause DNA damage. In a previous investigation, possible mechanisms of photogenotoxic

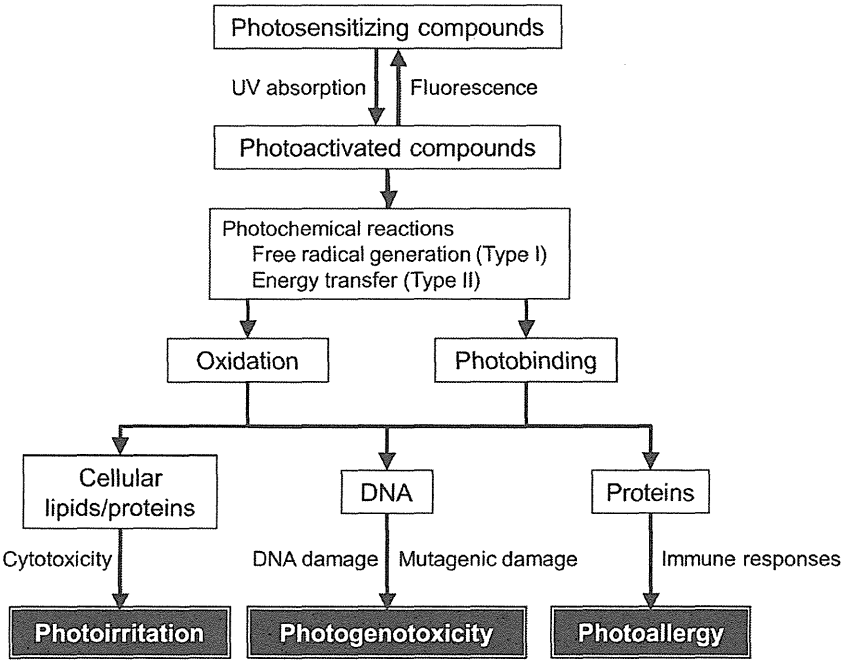

Fig. (1). Schematic representation of possible pathways for phototoxic reactions by chemicals.

reactions were deduced that DNA damage occurred after interaction of photosensitizer with DNA, such as intercalation of photosensitizer to double-stranded DNA, followed by direct reactions of photoactivated chemicals with DNA or ROS-mediated indirect reactions [20].

2.3. Photoallergy

Photoallergy is an immune response to photosensitizer-bound proteins in the skin and eyes. In the photoallergic pathway, photosensitizers play a role as a hapten and form an antigen with a protein, and then the antigen is presented by epidermal Langerhans cells to immunocompetent cells [21]. Antigenic photosensitizer-protein complexes could be formed mainly through two major pathways, and the photosensitizers were described as photohaptens and prohaptens [22]. Photohaptens covalently couple with carrier proteins *via* radical reactions under UV irradiation although they bind non-covalently to carrier proteins before exposure to UV. Prohaptens are converted to complete haptens by exposure to UV radiation, and then the prohaptens bind to carrier proteins.

3. ASSESSMENTS ON PHOTOSAFETY

In a clinical setting, measurements of minimal erythemal doses [23], oral provocation tests, and photopatch test [24] have been conducted for evaluating drug-induced phototoxicity on the basis of both photoirritant and photoallergic reactions in the skin. In drug discovery, an *in vitro* 3T3 neutral red uptake phototoxicity test (3T3 NRU PT) has been used as a simplified alternative approach for estimating the photosafety of chemicals [25]; however, the *in vitro* 3T3 NRU PT has some drawbacks, such as too many false-positive results and low throughput. Higher-throughput and different pathogenesis-based screening systems might be useful for precise phototoxic evaluation in an early phase of drug discovery. On the basis of the pathogenesis of drug-induced phototoxicity, various assessment tools for evaluating phototoxic potentials of chemicals have been developed over the past few years (Table 1). However, these phototoxic assessment tools cannot always be applied to all types of new drug substances owing to the physicochemical problems of tested chemicals and the limited specificity and sensitivity of the screening systems, so that researchers require a thorough understanding of the limitations on each assessment. The photosafety assay systems should be designed for intended types of phototoxic responses such as photoirritation, photogenotoxicity and/or photoallergy.

3.1. *In Silico* Prediction

In silico prediction systems, such as deductive estimation of risk from existing knowledge (DEREK) and highest

Table 1. Assay Systems for Evaluating Phototoxic Risk of Chemicals

Assessments		References
In silico prediction	DEREK	Barratt *et al.* [26, 27]
	HOMO-LUMO gap	Betowski *et al.* [28]
	QSAR model	Ringeissen *et al.* [29]
Photochemical properties	UV absorption	Henry *et al.* [34]
	ROS assay	Onoue *et al.* [13, 31]
	D-ROM assay	Onoue *et al.* [32]
Phototoxicity/photoirritancy	Photopatch test	Epstein [24]
	Photo-basophil-histamine-release test	Przybilla *et al.* [9]
	Photohemolysis model	Selvaag *et al.* [36], Sugiyama *et al.* [37]
	Human reconstituted epidermis model	Portes *et al.* [38]
	3T3 NRU phototoxicity test	Spielmann *et al.* [25]
	Oxygen consumption in *Bacillus subtilis*	Beani *et al.* [41]
	Yeast growth inhibition assay	Sugiyama *et al.* [42]
Photogenotoxicity	Photo Ames test	Brendler-Schwaab *et al.* [18], Marrot *et al.* [43]
	Photo chromosomal aberration test	Brendler-Schwaab *et al.* [18]
	Photo comet assay	Brendler-Schwaab *et al.* [18], Flamand *et al.* [44]
	Capillary gel-electrophoretic DNA-photocleaving assay	Onoue *et al.* [48]
	DNA-binding assay	Onoue *et al.* [20]
	IBP assay	Seto *et al.* [49]
Photoallergy	Pig skin model	Sarabia *et al.* [50]
	Photo-h-CLAT	Hoya *et al.* [51]
	Local lymph node assay	Vohr *et al.* [58]

occupied molecular orbital-lowest unoccupied molecular orbital (HOMO-LUMO) gap, can help to predict the phototoxic risk of chemicals before chemicals are synthesized. DEREK allows toxicity prediction of chemicals based on structures associated with the incidence of toxicity [26, 27], and the HOMO-LUMO gap provides photoreactive potentials of chemicals using energy differences between levels of HOMO and LUMO [28]. Recently, quantitative structure-activity relationship (QSAR) models have been proposed to predict phototoxic effects [29, 30]. These *in silico* prediction methods would help to reduce the various resources required for the synthesis of new drug candidates.

3.2. Photochemical Evaluation

After chemicals are newly synthesized, examination of their photochemical properties would be useful for phototoxic evaluation. UV spectral analysis provides information on the photoexcitability of chemicals on the basis of the first law of photochemistry. From the results from UV absorption, the photoexcitability of chemicals can be obtained. However, the true photoreactivity of chemicals, possibly leading to a phototoxic reaction, is unclear because some excited chemicals can release the absorbed photon energy *via* emission of fluorescence, phosphorescence and heat, returning to the ground state.

On the basis of the pathogenesis of drug-induced phototoxicity, a ROS assay and derivatives of reactive oxygen metabolites (D-ROM) assay were proposed for evaluating the phototoxic risk of chemicals. The ROS assay can monitor generation of ROS, such as singlet oxygen and superoxide, from photoirradiated chemicals, and the ROS data can be used to evaluate the photoreactivity of chemicals [13, 31]. The D-ROM assay can also determine ROS generation from photoirradiated chemicals *via* monitoring of the generation of ROS-derived metabolites, and the D-ROM assay can shorten irradiation time compared with that of the ROS assay [32]. In theory, the ROS assay would capture all the photochemically active substances; however, there is the probability that, as well as the phototoxins, some photolabile substances would also be recognized to be phototoxic by the ROS assay, because of significant ROS generation during the photodegradation processes. Namely, the ROS assay sometimes provide falsely positive predictions, and better understanding of the limitation would be of great help for avoiding the overestimation and misleading conclusion.

In addition, Kleinman and co-workers demonstrated that photostability data on chemicals would also be a good predictor of phototoxicity [33]. In contrast, Henry and co-workers suggested that photostability testing in physiological buffers were inadequate predictors of possible photosafety liabilities and should not be used solely to make photosafety risk assessments for new chemical entities [34]. Thus, usefulness of photostability data for photosafety evaluation is a matter of controversy.

These screening systems can comprehensively evaluate the phototoxic potentials of chemicals, such as photoirritation, photogenotoxicity and photoallergy, with high throughput, and they may be appropriate for evaluating the phototoxic risk of chemicals as a 1st screening in an early stage of drug discovery.

3.3. *In Vitro* Phototoxic Assessments

As photoirritant risk assessment tools, the 3T3 NRU PT are practically used for a hazard identification. The *in vitro* 3T3 NRU PT is a highly sensitive methodology for evaluating phototoxic risk, especially photoirritant risk, and contributes to the development of drug candidates; however, the photosafety assessment of chemicals by 3T3 NRU PT sometimes provides false positives because of its high detection sensitivity [35]. Additionally, UVB radiation is generally attenuated in the 3T3 NRU PT because cells were killed by UVB radiation; therefore, chemicals excited by only UVB exposure produce false-negative results in the assay. Other assay systems based on the release of inflammatory mediators from leucocytes (photo-basophil-histamine-release test) [9], membrane damage of erythrocytes (photohemolysis model) [36, 37], morphological change of three-dimensional human skin model (human reconstituted epidermis model) [38-40], change of oxygen consumption in *Bacillus subtilis* (oxygen consumption in *Bacillus subtilis*) [41] and growth inhibition of yeast cells (yeast growth inhibition assay) [42] would also be useful for *in vitro* photoirritation assessments. In particular, human reconstituted epidermis model has been thought as a suitable 3-D *in vitro* tool to predict phototoxicity of test chemicals intended for topical use [38-40].

For evaluating the photogenotoxic potential of chemicals, a photo Ames test has mainly been used, the mechanism of which is based on mutations in genes involved in histidine synthesis [18, 43], and a photo comet assay is also conducted for evaluating *in vivo*/*in vitro* photogenotoxic potentials, which is a sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell [18, 44, 45]. Photo chromosomal aberration test using several cell lines, including Chinese hamster fibroblasts and primary human lymphocytes, has been regarded as the assessment of a photocarcinogenic potential [18]. As a cell-free screening system, DNA-photocleaving assay has been widely used for evaluating photochemical genotoxicity [46, 47]. Onoue and co-workers also proposed three cell-free photogenotoxicity assessment tools based on DNA damage monitored by electrophoresis and DNA intercalator. A capillary gel-electrophoretic DNA-photocleaving assay monitors photocleavage of plasmid DNA by photoirradiated chemicals [48]. A DNA-binding assay evaluates the DNA-binding affinity of chemicals on the basis of results from ethidium displacement or fluorescence titration experiments, and the assay could be used as a 2nd screening following the ROS assay for evaluating the photogenotoxic risk of compounds [20]. An intercalator-based photogenotoxicity (IBP) assay monitors DNA damage by photoirradiated chemicals on the basis of fluorescence emission from DNA-intercalator complexes [49].

A pig skin model and a photo human cell line activation test (photo-h-CLAT) were developed for evaluating the photoallergic potential of chemicals. The pig skin model evaluates the formation of drug-protein photoadduct in pig skin [50], and the photo-h-CLAT monitors the overexpression of CD86/54 on the dendritic cells in antigen presentation to T lymphocytes [51]. Although the assays have not been internationally accepted, they might be

effective for evaluating the photoallergic potential of compounds because of the assay sensitivity and prediction capacity.

3.4. *In Vivo* Phototoxic Assessments

In addition to the *in vitro* phototoxicity screening systems, animal phototesting would be effective for identifying phototoxic potential of new drug candidates before human studies [52]. Human phototoxins have been detected in several animal studies using rabbits, hairless mice and guinea pigs [53], in which a phototoxic responses is determined upon a subjective evaluation of erythema and oedema at the irradiated sites as compared with non-irradiated sites. In addition to these photosafety assessments, sunburn cell counting [54], a mouse skin swelling method [55], a mouse tail swelling method [56] and a mouse ear swelling method [53] were developed, and these animal methods have been commonly used to test for acute phototoxic potential of chemicals in the pharmaceutical development. Colorimetrical evaluation on dermal surface before and after UV-exposure would also be of great use for photosafety assessment, since coloring of the skin might reflect the photobiological reactions of chemicals on the skin surface [57]. For evaluation of the photoallergic potential, local lymph node assay (LLNA) was developed, in which the immune reactions in the draining lymph nodes were measured [58]. However, outcomes from these animal phototestings would be affected by several factors, including the species, light source and dose, and the exposure of tested chemicals depending on the dosage form, route of administration and dosing regimen [59].

4. PHOTOSAFETY ASSESSMENT STRATEGIES

The photosafety assessment tools are effective for evaluating the photosafety of chemicals and for clarifying the mechanism of phototoxicity for chemicals in detail; however, the results from *in vitro* photosafety evaluations do not always reflect clinical observations on drug-induced phototoxicity. Phototoxic reactions mainly occur in the skin after topical and systemic administration; therefore, further characterization of the skin distribution properties of chemicals would be essential for reliable photosafety prediction. Previously, Seto and co-workers proposed a screening strategy for evaluating the *in vivo* phototoxicity of chemicals on the basis of their (i) photochemical characteristics, (ii) *in vitro* phototoxic properties and (iii) *in vivo* pharmacokinetic (PK) behaviors (Fig. **2**), and the screening strategy could improve the clinical relevance of *in vitro* photosafety evaluations on chemicals [57]. A discrete PK study was originally used in the screening strategy; however, a discrete PK study is time- and resource-intensive because a large number of drug candidates have to be examined in drug discovery. In particular, a large number of animals have to be sacrificed to obtain sufficient data on drug candidates in the discrete PK approach. To overcome these drawbacks of discrete PK study, a cassette dosing PK approach was applied to the photosafety assessment strategy [60]. The combined use of *in vitro* photochemical/phototoxic and cassette dosing PK data may enable evaluation and comparison of *in vivo* phototoxic risk of multiple compounds with high clinical relevance and high throughput. However, there is no denying that cassette dosing approach can cause multi-drug interaction, resulting in different outcomes from single-drug assessment methods. In this context, careful consideration on experimental conditions and chemical suitability should be made in order not to provide false negative predictions.

In addition to photobiochemical and *in vitro* phototoxic data, skin and eye distribution properties of chemicals are also important for exhaustive photosafety assessments, and

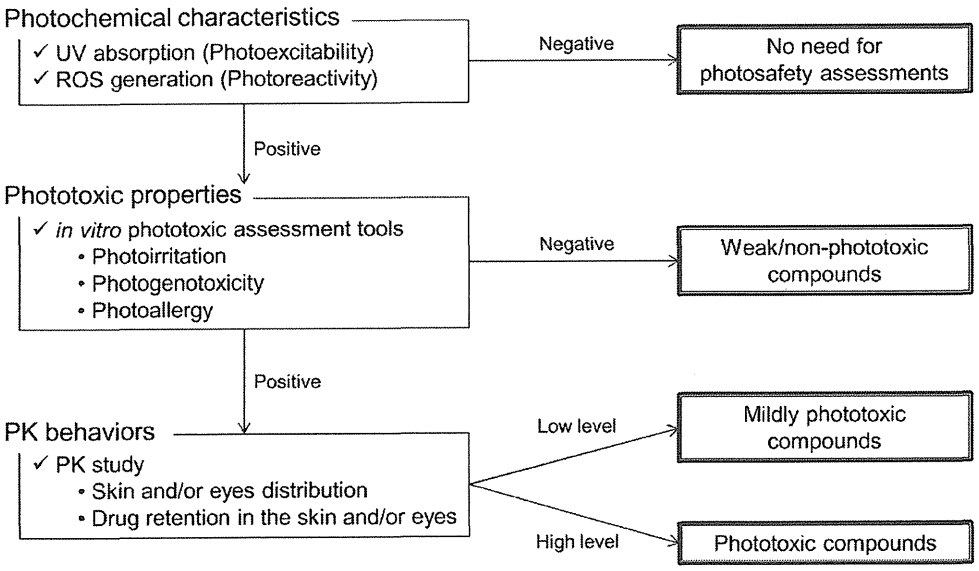

Fig. (2). Screening strategy of photosafety assessments for new drug candidates. Compounds are initially examined for their photoreactivity, and then the photoreactive chemicals are characterized in terms of *in vitro* phototoxic properties. The phototoxic chemicals are also subjected to PK study. The compounds would have potent *in vivo* phototoxic risk if they are distributed and retained at high levels in skin and eyes after topical or systemic administration.

Table 2. Guidelines for Evaluating Phototoxicity of Drug Candidates

Organization		EMEA/CPMP	FDA/CDER	OECD
Guidelines		Guidance on photosafety testing	Guidance for industry photosafety testing	*In vitro* 3T3 NRU phototoxicity test
Scope of test chemicals		Chemical substances Biotechnology-based drugs	All active ingredients and ingredients of preparations (In the case of external application, use of preparations is recommended.)	Chemical substances absorbing UV/VIS
UV/VIS absorption properties	Range	290–700 nm	290–700 nm	290–700 nm
	MEC	No description	No description	More than 10 $M^{-1} \cdot cm^{-1}$
Administration routes and internal distribution		Topical application Reaching the skin/eyes	Application to the skin/eyes Distribution in the skin/eyes Affecting the skin/eyes	No description

the screening strategy may enable the provision of reliable assessments on the *in vivo* phototoxic risk of chemicals and aid productive drug development. Furthermore, comparing the PK parameters of chemicals among different types of administration might enable appropriate administration to be identified and avoid or modulate the phototoxic risk of compounds.

5. GUIDANCE FOR PHOTOSAFETY ASSESSMENTS OF CHEMICALS

In the early 2000s, the regulatory agencies in the US and EU published guidelines for photosafety assessments of drug candidates (Table **2**). The European Medicines Agency (EMEA)/Committee for Proprietary Medicinal Products (CPMP) issued guidance on photosafety testing in 2002 [6], and the Food and Drug Administration (FDA)/Center for Drug Evaluation and Research (CDER) also published guidance for industry photosafety testing in 2003 [5]. The EMEA also published a concept paper in 2008 [61], which proposes a testing strategy that attempts to merge the testing proposals recommended by the FDA and the EMEA. The Organisation for Economic Co-operation and Development (OECD) presented guideline on phototoxic assessment tools in 2004 [7], and the *in vitro* 3T3 NRU PT was recommended in these guidelines as a validated methodology for evaluating the phototoxic potential of chemicals.

In these guidelines, chemicals, administered to the skin and/or eyes and distributed to the skin and/or eyes after administration, need to be examined for their phototoxic potential. The Grotthuss-Draper law states that light must be absorbed by a compound in order for photochemical reactions to take place. On the basis of this principle, all the guidelines have suggested that the phototoxic potential of chemicals is related to the photochemical properties of compounds, especially light absorption properties within 290–700 nm, and they have described the need for measurement of the light absorption properties of chemicals as a first screening. Not all of the guidelines have provided specific levels on the chemical concentration in the skin and/or eyes, and only the OECD guidelines have stated the criterion of a molar extinction coefficient (MEC) at 10 $M^{-1} \cdot cm^{-1}$ for deciding whether photosafety testing on chemicals is needed. However, the criterion would practically impel developers to conduct photosafety testing on almost all newly synthesized chemicals in drug discovery, and the more specific criteria of MEC should be set for deciding on whether to conduct photosafety testing. Henry *et al.* measured UV absorption spectra ranging from 290 to 700 nm on 35 phototoxic compounds [34], and proposed that photosafety testing on compounds might not be needed if the MEC of a compound is estimated as less than 1,000 $M^{-1} \cdot cm^{-1}$ in UV absorption spectral analysis. However, UV absorption of chemicals would not always correlate with their phototoxic potential directly, so combination use of MEC approach and other appropriate screening system might be advantageous for avoiding false predictions. Currently, other screenings based on physicochemical properties, such as photostability testing and structural alerts using DEREK, have been anticipated to become efficacious tools for deciding whether photosafety testing is needed.

If there is a possibility of phototoxic potential, further phototoxicity tests, including photoirritation, photoallergy and photogenotoxicity, should be conducted. However, the EMEA and International Workshop on Genotoxicity Tests (IWGT) no longer recommend photogenotoxicity testing as part of standard photosafety evaluation [62]. The OECD guideline has recommended an *in vitro* 3T3 NRU PT and set the specific criteria for evaluating whether chemicals have phototoxic risk. The assay was drafted and submitted to the OECD as an alternative method for *in vivo* phototoxicity testing by the European Centre for the Validation of Alternative Methods (ECVAM) and the European Cosmetics, Toiletry and Perfumery Association (COLIPA) [25]. The *in vitro* 3T3 NRU PT sometimes provides false-positive results, and the results from the assessments would not always reflect other types of *in vitro* phototoxic risk, including photogenotoxicity and photoallergy, as well as *in vivo* phototoxicity. Thus, inclusive *in vitro* screening methodologies and strategies would also be needed for more reliable phototoxic evaluation.

Validation study on the ROS assay has been carried out by the Japan Pharmaceutical Manufacturers Association (JPMA), supervised by the Japanese Center for the Validation of Alternative Methods (JaCVAM), to verify relevance and reliability. Currently, in the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), introduction of the validated ROS assay in the photosafety guidelines is under consideration (ICHS10 Guideline: "Photosafety Evaluation of Pharmaceuticals").

6. CONCLUSION

To avoid drug-induced phototoxicity, researchers, mainly in the US and EU, have developed photosafety assessment tools, and the *in vitro* 3T3 NRU PT, validated by the ECVAM and the COLIPA and listed in the OECD guideline, has been widely used as an effective photosafety assessment tool. Recently, the ICH has taken up photosafety evaluation of pharmaceutics as a new topic, and the ROS assay has been discussed in the ICH as a new photosafety assessment tool. Validation study of the ROS assay has been undertaken by the JPMA and the JaCVAM. Although simplified screenings are effective for evaluating the *in vitro* photosafety of drug candidates in an early phase of drug discovery, no internationally standardized screening strategy for evaluating the *in vivo* photosafety of chemicals has been established. For further characterization, use of PK behaviors for chemicals, such as their skin and eye distribution and skin and eye retention properties, would be efficacious as additional parameters for photosafety evaluation, and the combined use of *in vitro* photobiochemical/phototoxic and PK data might enable evaluation of *in vivo* phototoxic risk with high clinical relevance. Interest on the photosafety of chemicals would increase in both regulatory sciences and drug discovery, and the development of effective screening strategies for evaluating the phototoxic risk of chemicals would provide reliable photosafety assessments and aid productive research and development of new pharmaceuticals.

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CONFLICT OF INTEREST

Declared none.

ABBREVIATIONS

3T3 NRU PT	=	3T3 neutral red uptake phototoxicity test
CDER	=	Center for Drug Evaluation and Research
COLIPA	=	European Cosmetics, Toiletry and Perfumery Association
CPMP	=	Committee for Proprietary Medicinal Products
DEREK	=	Deductive estimation of risk from existing knowledge
D-ROM	=	Derivatives of reactive oxygen metabolites
ECVAM	=	European Centre for the Validation of Alternative Methods
EMEA	=	European Medicines Agency
FDA	=	Food and Drug Administration
HOMO	=	Highest occupied molecular orbital
IBP	=	Intercalator-based photogenotoxicity
ICH	=	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
IWGT	=	International Workshop on Genotoxicity Tests
JaCVAM	=	Japanese Center for the Validation of Alternative Methods
JPMA	=	Japan Pharmaceutical Manufacturers Association
LUMO	=	Lowest unoccupied molecular orbital
MEC	=	Molar extinction coefficient
NSAIDs	=	Non-steroidal anti-inflammatory drugs
OECD	=	Organisation for Economic Co-operation and Development
photo-h-CLAT	=	Photo human cell line activation test
PK	=	Pharmacokinetic
ROS	=	Reactive oxygen species
UV	=	Ultraviolet
VIS	=	Visible

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