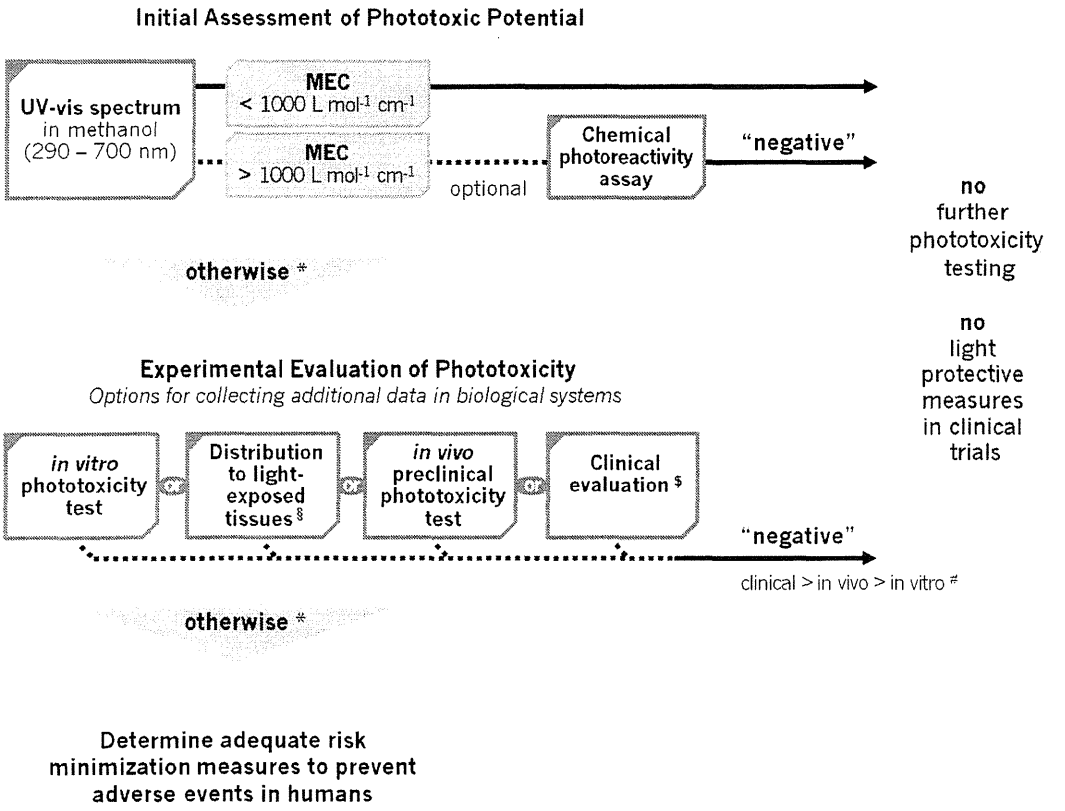
photochemical properties and pharmacological/chemical class be undertaken before outpatient studies. Characterization of the UV-visible absorption spectrum is recommended as the initial assessment because it can obviate any further photosafety evaluation. In addition, the distribution to skin and eye can be evaluated to inform further on the human risk and the recommendations for further testing. Then, if appropriate, an experimental evaluation of phototoxicity potential (*in vitro* or *in vivo*, or clinical) should be undertaken before exposure of large numbers of subjects (Phase 3).

Figure 1 provides an outline of possible phototoxicity assessment strategies. The figure is based on the strategies outlined in this section of this document. The strategies are flexible. Depending on the particular situation, some portions of the assessment are optional and might not be conducted.

Figure 1. Outline of possible phototoxicity assessment strategies for pharmaceuticals given *via* systemic and dermal routes



* "otherwise": data do not support a low potential for phototoxicity or have not been generated (assay/test/evaluation not conducted)

\# A "negative" result in an appropriately conducted *in vivo* phototoxicity study supersedes a positive *in vitro* result. A robust clinical phototoxicity assessment indicating no concern supersedes any positive nonclinical results. A positive result in an *in vitro* phototoxicity test could also, on a case-by-case basis, be negated by tissue distribution data (see text). In the United States, for products applied dermally, a dedicated clinical trial for phototoxicity on the to-be-marketed formulation can be warranted in support of product approval.

$ Clinical evaluation could range from standard reporting of adverse events in clinical studies to a dedicated clinical photosafety trial.

§ Tissue distribution is not a consideration for the phototoxicity of dermal products.

5.1. Recommendations for Pharmaceuticals Given *via* Systemic Routes

5.1.1 Assessment of Phototoxicity Potential

If the substance does not have a MEC greater than 1000 L mol^{-1} cm^{-1} (between 290 and 700 nm), no photosafety testing is recommended and no direct phototoxicity is anticipated in humans. However, it should be noted that phototoxicity by indirect mechanisms (e.g., pseudoporphyria or porphyria), although rare, could still occur. For compounds with MEC values of 1000 L mol^{-1} cm^{-1} or higher, if the drug developer chooses to conduct a test for photoreactivity a negative result could support a decision that no further photosafety assessment is warranted (see Section 3.2). Otherwise, nonclinical and/or clinical photosafety assessment of the substance should be conducted. Available data on the phototoxicity of chemical class-related compounds should be evaluated as this could inform on the approach to be taken.

5.1.2 Experimental Evaluation of Phototoxicity

In order to reduce the use of animals in accordance with the 3R principles, a validated *in vitro* method should generally be considered before conducting animal testing (e.g., see Directive 2010/63/EU). If the drug developer chooses an *in vitro* approach, the 3T3 NRU-PT is currently the most widely used assay and in many cases could be considered as an initial test for phototoxicity. The high sensitivity of the 3T3 NRU-PT results in good negative predictivity, and negative results are generally accepted as sufficient evidence that a substance is not phototoxic. In such cases no further testing is recommended and no direct phototoxicity is anticipated in humans.

In some situations (e.g., poorly soluble compounds) an initial assessment of phototoxicity in an *in vitro* assay might not be appropriate. In this case, an assessment in animals or in humans could be considered. Alternatively, if drug distribution data are available, they could, on a case-by-case basis, support a decision that no further photosafety assessment is warranted (see Section 2.2).

If an *in vitro* phototoxicity assay gives a positive result, a phototoxicity study in animals could be conducted to assess whether the potential phototoxicity identified *in vitro* correlates with a response *in vivo*. Alternatively, drug distribution data could, on a case-by-case basis, support a position that the risk of phototoxicity *in vivo* is very low and that no further photosafety assessment is warranted (see Section 2.2). As another option, the photosafety risk could be assessed in the clinical setting, or managed by the use of light-protective measures. A negative result in an appropriately conducted phototoxicity study either in animals or humans supersedes a positive *in vitro* result. In such cases no further testing is recommended and no direct phototoxicity is anticipated in humans.

A positive result in an *in vivo* animal study can, in certain circumstances, be mitigated using a NOAEL-based risk assessment, typically considering C_{max} comparisons. Otherwise, a clinical assessment is warranted. In all cases a robust clinical phototoxicity assessment indicating no concern supersedes any positive nonclinical results.

A positive result in an *in vitro* phototoxicity test would not be negated by a negative result in a subsequently conducted chemical photoreactivity assay (e.g., a ROS assay).

In cases where an animal or clinical phototoxicity study has already been conducted, there is no reason to subsequently conduct either a chemical photoreactivity or an *in vitro* phototoxicity assay.

5.2. Recommendations for Pharmaceuticals Given *via* Dermal Routes

5.2.1 Assessment of Phototoxicity Potential

If the active substance and excipients do not have MEC values greater than 1000 L mol^{-1} cm^{-1} (between 290 and 700 nm), no further photosafety testing is recommended and no phototoxicity is anticipated in humans. For compounds with MEC values of 1000 L mol^{-1} cm^{-1} or higher, negative photoreactivity test results (e.g., a ROS assay) can support a decision that no further photosafety assessment is warranted (see Note 5 for exception). If further assessment is warranted, available data on the phototoxicity of chemical class-related compounds should be evaluated, as this could inform on the approach to be taken.

Tissue distribution is not a consideration for the phototoxicity of dermal products. Dermal products are administered directly to the skin and hence, unless they are applied to areas not usually exposed to light, are assumed to be present in light-exposed tissues.

5.2.2 Experimental Evaluation of Phototoxicity and Photoallergy

The 3T3 NRU-PT can be used to assess individually the phototoxicity potential of the API and any new excipient(s), provided that appropriate testing conditions can be achieved (e.g., test concentrations not limited by poor solubility, relevant UVB dose can be applied). In cases where no phototoxic component has been identified *in vitro*, the overall phototoxicity potential of the clinical formulation can be regarded as low.

Some properties of the clinical formulation that could influence the potential phototoxic response (e.g., penetration into skin, intracellular uptake) cannot be evaluated using the 3T3 NRU-PT alone. Therefore, confirmation of the overall negative result in an evaluation using the clinical formulation and/or monitoring during clinical trials can still be warranted.

Reconstructed human skin models can be used to assess the phototoxicity potential of clinical formulations. Under adequate test conditions (see Section 3.3), a negative result in a reconstructed human skin assay indicates that the direct phototoxicity potential of the formulation can be regarded as low. In this case, generally no further phototoxicity testing is recommended (see Note 5 for exception).

If an appropriate *in vitro* assay is not available, the initial test could be an *in vivo* phototoxicity test on the clinical formulation. A negative result in an appropriately conducted *in vivo* animal phototoxicity study would be sufficient evidence that the formulation is not directly phototoxic and no further phototoxicity testing is recommended (see Note 5 for exception). Alternatively, the phototoxicity potential can be assessed in the clinical setting.

For dermal products where the API or any new excipient has a MEC value greater than 1000 L mol^{-1} cm^{-1} at any wavelength between 290 and 700 nm, a photoallergy assessment is generally warranted in addition to phototoxicity testing. As the predictivity of nonclinical photoallergy tests is unknown, this would typically be a clinical assessment using the to-be-marketed formulation and conducted during Phase 3.

Photosafety evaluation of the clinical formulation delivered *via* dermal patches can follow the above described principles for clinical dermal formulations. For transdermal patches, the principles for both dermal and systemic drugs should be applied. In addition, the intended clinical use (e.g., skin area recommended for use, duration of application) and the properties of the patch matrix (e.g., being opaque to UV and visible light) should be considered for the overall risk assessment.

6. ENDNOTES

Note 1 For compounds that absorb at relevant wavelengths, have a MEC value greater than 1000 L mol^{-1} cm^{-1}, and are given *via* ocular routes (e.g., eye drops, intraocular injections), an evaluation of the phototoxicity potential should be undertaken in accordance with the general principles of phototoxicity assessment. Biodistribution of drug in the eye, and optical properties of the eye should also be considered. Any available information on the compound or chemical class-related compounds should be considered in the overall assessment.

Compounds that only absorb light at wavelengths below 400 nm and are to be administered as intraocular injections behind the lens (e.g., in the vitreous) are of low concern for retinal phototoxicity, as only light of wavelengths greater than 400 nm reaches the back of the adult eye. However, the lens in children of less than approximately 10 years of age is not completely protective against wavelengths below 400 nm.

Note 2 Testing for photogenotoxicity is not recommended as a part of the standard photosafety testing program. In the past, some regional guidelines (e.g., CPMP/SWP/398/01) have recommended that photogenotoxicity testing be conducted, preferentially using a photoclastogenicity assay (chromosomal aberration or micronucleus test) in mammalian cells *in vitro*. However, experience with these models since the CPMP/SWP guideline was issued has indicated that these tests are substantially oversensitive and even incidences of pseudo-photoclastogenicity have been reported (Ref. 8). Furthermore, the interpretation of photogenotoxicity data regarding its meaning for clinically relevant enhancement of UV-mediated skin cancer is unclear.

Note 3 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). Methanol is recommended as a preferred solvent and was used to support the MEC threshold of 1000 L mol^{-1} cm^{-1} (Ref. 3). When measuring UV-visible light spectra, potential limitations (e.g., artifacts due to high concentrations or low solubility, including slow precipitation) should be considered. If the chromophore of the molecule appears to be pH-sensitive (e.g., phenolic structure, aromatic amines, carboxylic acids, etc.) an additional spectrum obtained under aqueous, pH 7.4-buffered conditions, could add valuable information regarding differences in the shape of the absorption spectrum and in the MECs. If significant differences are seen between measurements obtained in methanol versus pH-adjusted conditions, the MEC threshold of 1000 L mol^{-1} cm^{-1} cannot be used to obviate further photosafety assessment.

Note 4 A survey of pharmaceutical companies indicated that the 3T3 NRU-PT, as described in Organisation for Economic Co-operation and Development, Test Guideline (OECD TG) 432, generates a high percentage of positive results (approximately 50%), the majority of which do not correlate with phototoxicity responses in animals or humans (Ref. 9). Following a retrospective review of data for pharmaceuticals, a reduction of the maximum test concentration from 1000 to 100 µg/mL appears justified (Ref. 10). Compounds without any significant cytotoxicity (under irradiation) up to this limit can be considered as being devoid of relevant phototoxicity. In addition, the category named "probable phototoxicity" per OECD TG 432 (i.e., Photo Irritation Factor (PIF)

values between 2 and 5 or Mean Photo Effect (MPE) values between 0.10 and 0.15) is of questionable toxicological relevance for systemic drugs. Compounds in this category generally do not warrant further photosafety evaluations. For compounds with a PIF value between 2 and 5, and for which it is not possible to determine an IC_{50} in the absence of irradiation, it is important to check that the compound is not classified as positive using the MPE calculation, i.e., that the MPE is less than 0.15.

Systemic drugs that are positive in the 3T3 NRU-PT only at *in vitro* concentrations that are many times higher than drug concentrations likely to be achieved in light-exposed tissues in humans, can, on a case-by-case basis, and in consultation with regulatory authorities, be considered to be 'low risk' for phototoxicity in humans , without follow-up *in vivo* testing.

Note 5 In the United States, for products applied dermally, a dedicated clinical trial for phototoxicity (photoirritation) on the to-be-marketed formulation (API plus all excipients) can be warranted in support of product approval.

7. GLOSSARY

3T3 NRU-PT:

In vitro 3T3 Neutral Red Uptake Phototoxicity Test.

Assessment:

In the context of this document, an assessment is an evaluation of all available information and does not always mean an additional test is conducted.

Chromophore:

The substructure of a molecule that absorbs visible or ultraviolet light.

Dermal Drugs:

Products applied topically to the skin.

Direct Phototoxicity:

Phototoxicity induced by absorption of light by the drug or excipient.

Indirect Phototoxicity:

Phototoxicity due to cellular, biochemical or physiological alterations caused by the drug or excipient, but not related to photochemical reactivity of the drug or excipient (e.g., perturbation of heme homeostasis).

Irradiance:

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

Irradiation:

The process by which an object/subject is exposed to UV or visible radiation.

MEC:

Molar Extinction Coefficient (also called molar absorptivity) reflects the efficiency with which a molecule can absorb a photon at a particular wavelength (typically expressed as $L\ mol^{-1}\ cm^{-1}$) and is influenced by several factors, such as solvent.

MPE:

The Mean Photo Effect is calculated for results of the 3T3 NRU-PT. The MPE is based on comparison of the complete concentration response curves (see OECD TG 432).

NOAEL:

No Observed Adverse Effect Level.

OECD TG:

Organisation for Economic Co-operation and Development, Test Guideline.

Outpatient Study:

A clinical study in which patients are not restricted to a clinical site.

Photoproducts:

New compounds/structures formed as a result of a photochemical reaction.

Photoreactivity:

The property of chemicals to react with another molecule as a consequence of absorption of photons.

PIF:

Photo Irritation Factor is calculated for results of the 3T3 NRU-PT by comparing the IC_{50} values obtained with and without irradiation.

ROS:

Reactive Oxygen Species, including superoxide anion and singlet oxygen.

Systemic drugs:

Products administered by a route that is intended to produce systemic exposure.

UVA:

Ultraviolet light A (wavelengths between 320 and 400 nm).

UVB:

Ultraviolet light B (wavelengths between 280 and 320 nm; as a part of sunlight wavelengths between 290 and 320 nm).

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別紙４

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yoshiki Seto, Masashi Kato, Shizuo Yamada, Satomi Onoue	Development of micellar reactive oxygen species assay for photosafety evaluation of poorly water-soluble chemicals	*Toxicology in Vitro*	27(6)	1838-1846	2013
Satomi Onoue, Gen Suzuki, Masashi Kato, Morihiko Hirota, Hayato Nishida, Masato Kitagaki, Hirokazu Kouzuki, Shizuo Yamada	Non-animal photosafety assessment approaches for cosmetics based on the photochemical and photobiochemical properties	*Toxicology in Vitro*	27(8)	2316-2324	2013
Satomi Onoue, Shizuo Yamada	Pirfenidone in respirable powder form for the treatment of pulmonary fibrosis: a safer alternative to the current oral delivery system?	*Therapeutic Delivery*	4(8)	887-889	2013
Satomi Onoue, Masashi Kato, Shizuo Yamada	Development of an albuminous reactive oxygen species assay for photosafety evaluation under experimental biomimetic conditions	*Journal of Applied Toxicology*	34(2)	158-165	2014
Satomi Onoue, Kazuhiro Hosoi, Tsuguto Toda, Hironori Takagi, Naoto Osaki, Yasuhiro Matsumoto, Satoru Kawakami, Shinobu Wakuri, Yumiko Iwase, Toshinobu Yamamoto, Kazuichi Nakamura, Yasuo Ohno, and Hajime Kojima	Intra-/inter-laboratory validation study on reactive oxygen species assay for chemical photosafety evaluation using two different solar simulators	*Toxicology in Vitro*	28	515-523	2014
Satomi Onoue, Masashi Kato, Ryo Inoue, Yoshiki Seto, Shizuo Yamada	Photosafety screening of phenothiazine derivatives with combined use of photochemical and cassette-dosing pharmacokinetic data	*Toxicological Sciences*	137(2)	469-477	2014

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Development of micellar reactive oxygen species assay for photosafety evaluation of poorly water-soluble chemicals

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ABSTRACT

A reactive oxygen species (ROS) assay was previously developed for photosafety assessment; however, the phototoxic potential of some chemicals cannot be evaluated because of their limited aqueous solubility. The present study was undertaken to develop a new micellar ROS (mROS) assay system for poorly water-soluble chemicals using a micellar solution of 0.5% (v/v) Tween 20 for solubility enhancement. In repeated mROS assay, intra- and inter-day precisions (coefficient of variation) were found to be below 11%, and the *Z'*-factors for singlet oxygen and superoxide suggested a large separation band between positive and negative standards. The ROS and mROS assays were applied to 65 phototoxins and 18 non-phototoxic compounds for comparative purposes. Of all 83 chemicals, 25 were unevaluable in the ROS assay due to poor solubility, but only 2 were in the mROS assay. Upon mROS assay on these model chemicals, the individual specificity was 76.5%, and the positive and negative predictivities were found to be 93.9% and 86.7%, respectively. The mROS assay provided 2 false negative predictions, although negative predictivity for the ROS assay was found to be 100%. Considering the pros and cons of these assays, strategic combined use of the ROS and mROS assays might be efficacious for reliable photosafety assessment with high applicability and predictivity.

1. Introduction

Several classes of pharmaceutics, cosmetics and food ingredients can be excited by sunlight, consisting of partial ultraviolet (UV) B (290–320 nm), UVA (320–400 nm) and visible light (400–700 nm); then, these photo-excited agents can elicit phototoxic reactions in skin and eyes (Epstein, 1983; Moore, 2002; Onoue et al., 2009). For photosafety evaluation, a number of effective *in vitro* methodologies have been proposed within the past few decades (Seto et al., 2012), and, notably, a UV absorption system (Henry et al., 2009) and a 3T3 neutral red uptake phototoxicity test (Spielmann et al., 1994) were recommended in the Organisation for Economic Co-operation and Development (OECD) guideline (OECD, 2004). Considering the implementation of the 3Rs principle (replacement, reduction and refinement), interest in the development of *in vitro* assessments based on photochemical and photobiological mechanisms should be increasing in photosafety assessments. A reactive oxygen species (ROS) assay was designed for the *in vitro* photoreactivity assessment of pharmaceuticals on the basis of ROS generation from photoirradiated chemicals, including singlet oxygen and superoxide (Onoue and Tsuda, 2006). The experimental conditions of the ROS assay were optimized (Onoue et al., 2008a,b) and validated (Onoue et al., in press), offering high assay productivity and prediction capacity.

Although the ROS assay demonstrated high prediction capacity for photosafety assessment, there appeared to be at least two assay limitations in a multi-laboratory validation study: (i) false positive predictions and (ii) solubility issues (Onoue et al., in press). Since the ROS assay is carried out in early phases of photosafety assessments, false positives would be re-evaluated by appropriate follow-up assessments. In this context, the former assay limitation might not be a severe problem. In contrast, the solubility issues would be a serious problem for reliable photosafety assessment. In the validation study (Onoue et al., in press), 43% of tested chemicals could not be dissolved in reaction mixtures at 200 μM owing to their poor water solubility, and, additional experiments on these chemicals had to be performed at lower concentrations (20 or 2 μM). The ROS data on some phototoxins at lower concentrations led to different observations among three laboratories, and ROS

Abbreviations: CV, coefficient of variation; DMSO, dimethyl sulfoxide; mROS assay, micellar reactive oxygen species assay; NaPB, sodium phosphate buffer; NBT, nitroblue tetrazolium; OECD, the Organisation for Economic Co-operation and Development; PABA, *p*-aminobenzoic acid; ROS, reactive oxygen species; SD, standard deviation; SDS, sodium dodecyl sulfate; UV, ultraviolet.

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data from chemicals at lower concentrations might not be suitable for photosafety assessment. Hence, appropriate modifications to the ROS assay system for enhanced applicability would be required for reliable photosafety assessment on poorly water-soluble chemicals.

For solubilizing poorly water-soluble drugs in oral and injectable solution forms, micelle systems are widely used in commercially available formulations (Strickley, 2004). In addition, a previous study demonstrated that the use of micellar solution systems, such as Tween 20, sodium laurate and sodium dodecyl sulfate (SDS), would be effective for monitoring singlet oxygen generation from poorly water-soluble chemicals because of the intense solubilizing potency and production of the biomembrane-mimetic environment (Onoue et al., 2008c). Thus, the present study attempted to develop a micellar ROS (mROS) assay with the aim of overcoming solubility issues of ROS assay, and thus a micellar solution of Tween 20 (polyoxyethylene sorbitan monolaurate), a non-ionic detergent, was applied to the ROS assay system. The precision and robustness of the mROS assay were evaluated by repeated measurement and calculation of Z'-factor, a parameter reflecting the quality of the assay. To verify the utility of the mROS assay, the number of evaluable compounds and the predictability for photosafety were compared between the ROS and mROS assays using 65 phototoxins and 18 non-phototoxic compounds.

2. Materials and methods

2.1. Chemicals

Amlodipine besylate (>98%; **5**), chlorpromazine HCl (>99%; **12**), ciprofloxacin (>98%; **14**), fenofibrate (>98%; **19**), fluvastatin Na (>98%; **21**), glibenclamide (>98%; **23**), gliclazide (>98%; **24**), griseofulvin (>95%; **25**), hydrochlorothiazide (>98%; **26**), ibuprofen (>98.5%; **27**), indomethacin (>98%; **28**), ketoprofen (>98%; **29**), lomefloxacin HCl (>98%; **31**), lovastatin (>95%; **33**), meloxicam (>98%; **35**), methotrexate (>98%; **36**), 6-methylcoumarin (>99%; **38**), mequitazine (>98%; **39**), nicardipine HCl (>99%; **42**), nitrendipine (>98%; **43**), norfloxacin (>98%; **44**), ofloxacin (>98%; **45**), omeprazole (>98%; **46**), piroxicam (>97%; **49**), pitavastatin Ca (>98%; **50**), pravastatin Na (>98%; **51**), promethazine HCl (>98%; **53**), anthracene (>99.5%; **62**), erythromycin (>98%; **68**), penicillin G (**69**), phenytoin (>98%; **70**), cinnamic acid (>99.5%; **74**), L-histidine (>98%; **76**), octrizole (>97%; **78**), *p*-aminobenzoic acid (PABA, 99.5–100.2; **81**), SDS (>99%; **82**), dimethyl sulfoxide (DMSO, >98%), imidazole (>98%), nitroblue tetrazolium (NBT, >98%), *p*-nitrosodimethylaniline (>97%), Tween 20 (2.2% water included), disodium hydrogen phosphate 12-water (>99%) and sodium dihydrogen phosphate dihydrate (>99%) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Rosiglitazone (>97%; **55**) and 4-methoxybenzylidene camphor (>99%; **77**) were purchased from Enzo Life Sciences International (Farmingdale, NY, USA) and Alfa Aesar (Ward Hill, MA, USA), respectively. Acridine HCl (>98%; **2**), bezafibrate (>98%; **9**), cilnidipine (>98%; **13**), clofibrate (>98%; **15**), naproxen (>99%; **41**), valsartan (>98%; **61**), avobenzone (>98%; **63**), hexachlorophene (>98%; **65**), benzocaine (>99%; **67**), sulisobenzone (>98%; **71**), octyl methoxycinnamate (>96%; **79**) and octyl salicylate (>98%; **80**) were bought from Tokyo Chemical Industry (Tokyo, Japan). Acridine (>97%; **1**), amiodarone HCl (>98%; **3**), benzbromarone (>95%; **8**), bufexamac (>98%; **10**), diclofenac Na (>98%; **16**), doxycycline HCl (>97%; **17**), fluphenazine 2HCl (>98%; **20**), furosemide (>98%; **22**), levofloxacin (>98%; **30**), 8-methoxypsoralen (>99%; **37**), nalidixic acid (>98%; **40**), perphenazine (**47**), quinine HCl (>99%; **54**), sparfloxacin (>98%; **56**), tamoxifen (>99%; **57**), tetracycline HCl (>95%; **58**), thioridazine HCl (>99%; **59**), bithionol (**64**), aspirin (>99%; **66**), bumetrizole (98%; **72**), chlorhexidine (>99.5%; **73**), drometrizole (97%; **75**) and UV-571 (>98%; **83**) were purchased from Sigma–Aldrich Japan (Tokyo, Japan). Amlodipine (>98%; **4**), atorvastatin (>99%; **6**), candesartan cilexetil (>99%; **11**), enoxacin (>98%; **18**), losartan K (>98%; **32**) and manidipine HCl (>99%; **34**) were obtained from LKT Laboratories (St. Paul, MN, USA). Benidipine HCl (>98%; **7**) was bought from Toronto Research Chemicals (Toronto, Ontario, Canada). Prochlorperazine dimaleate (>99%; **52**) and trifluoperazine (>99%; **60**) were purchased from MP Biomedicals (Santa Ana, CA, USA). Pirfenidone (>99%; **48**) was kindly provided by Shionogi (Osaka, Japan).

2.2. Irradiation conditions

Chemicals were stored in an Atlas Suntest CPS + solar simulator (Atlas Material Technology LLC, Chicago, USA) equipped with a xenon arc lamp (1500 W) and cooling unit SR-P20FLE (Hitachi, Tokyo, Japan). A UV special filter (#56052371, Atlas) 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. The irradiation test was carried out at 25 °C for 1 h with an irradiance of ca. 2.0 mW/cm^2 as determined using the calibrated UVA detector Dr. Hönle #0037 (Dr. Hönle, Munich, Germany).

2.3. Reactive oxygen species (ROS) assay

ROS assay was carried out for the detection of both singlet oxygen and superoxide generation as we reported previously (Onoue et al., 2008a; Onoue and Tsuda, 2006). Briefly, each tested compound was dissolved in DMSO at 10 mM for stock solution. To monitor the generation of singlet oxygen, samples containing compounds (200 μM), *p*-nitrosodimethylaniline (50 μM) and imidazole (50 μM) in 20 mM sodium phosphate buffer (NaPB, pH 7.4) were irradiated with simulated sunlight, and then the UV absorption at 440 nm was measured using SAFIRE (TECAN, Männedorf, Switzerland). For the determination of superoxide generation, samples containing the compounds (200 μM) and NBT (50 μM) in 20 mM NaPB (pH 7.4) were exposed to simulated sunlight, and the reduction of NBT was measured by the increase in the absorbance at 560 nm using SAFIRE. All samples were checked for precipitation by visual observation before and after light exposure. If the tested chemical was found to be insoluble in assay buffer, the assay could be carried out under appropriate dilution.

2.4. Micellar ROS (mROS) assay

Micellar solution of 0.5% (v/v) Tween 20 was applied to the ROS assay system. Critical micelle concentration (CMC) of Tween 20 was ca. 0.005% (v/v) in distilled water (Wan and Lee, 1974), and the applied concentration of Tween 20 for the mROS assay was almost identical to 100-fold of the CMC, offering high solubilizing potency. Briefly, to monitor the generation of singlet oxygen, compounds (200 μM), *p*-nitrosodimethylaniline (50 μM) and imidazole (50 μM) were dissolved in 20 mM NaPB (pH 7.4) with 0.5% (v/v) Tween 20. For the determination of superoxide generation, compounds (200 μM) and NBT (50 μM) were dissolved in 20 mM NaPB (pH 7.4) with 0.5% (v/v) Tween 20. Then, these samples were irradiated with simulated sunlight and measured in the same conditions with the ROS assay protocol. All samples were checked for precipitation by visual observation before and after light exposure. According to the results from preliminary study, Tween 20 was found to be weak ROS generator, so results were calculated by subtracting blank readings from sample readings.

2.5. Z′-factor

To evaluate the robustness of the mROS assay, Z′-factor, a statistical function, was calculated using the following equation: $Z' = 1 - (3\sigma c_+ + 3\sigma c_-)/|\mu_{c+} - \mu_{c-}|$ (Zhang et al., 1999). The means of positive and negative control signals are denoted as μ_{c+} and μ_{c-}, respectively. The standard deviations (SD) of positive and negative control signals are denoted as σ_{c+} and σ_c , respectively. The difference between the means, $|\mu_{c+} - \mu_{c-}|$, defined the assay dynamic range.

2.6. Criteria for data acceptance and judgment in the ROS or mROS assay

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

3. Results and discussion

3.1. Selection of detergent for mROS assay

On the basis of a previous study (Onoue et al., 2008c), the micellar solution system was applied to the ROS assay system with the aim of solubilizing poorly water-soluble chemicals. In general, superoxide can be detected by the conversion of NBT to monoformazan; however, a previous study demonstrated that the weak acidity at pH 6.2 attenuated the conversion from tetrazolium salt to formazan (Johno et al., 2010). Since the use of acidic detergents, including sodium laurate and SDS, might affect the determination of superoxide, a micellar solution of 0.5% (v/v) Tween 20, a nonionic detergent, was employed for the mROS assay.

3.2. Accuracy and precision

In this study, on the basis of previously reported photoreactivity and photosafety data (Seto et al., 2010), methotrexate (**36**) and erythromycin (**68**) were selected as positive and negative controls, respectively. The overall precisions of the ROS and mROS assays were compared by analyzing the photoreactivity of methotrexate (**36**) and erythromycin (**68**) at 200 μM. The intra-day precisions ($n = 9$) and inter-day precisions (days 1 and 2, $n = 18$) are shown in Table 1. The intra-day coefficients of variation (CV) for the detection of singlet oxygen and superoxide generation from irradiated methotrexate (**36**) in the ROS assay were found to be 9.4% and 3.6%, respectively, and those in the mROS assay were estimated to be 9.9% and 2.5%. The inter-day CV values for methotrexate (**36**) in the ROS assay were calculated to be 10.8% for singlet oxygen and 7.9% for superoxide, and those in the mROS assay were estimated to be 10.9% for singlet oxygen and 2.6% for superoxide. According to the obtained data, the mROS assay would have good intra-day and inter-day precisions, and they were almost identical to those of the ROS assay.

To assess the robustness and reproducibility of the micellar ROS assay, the Z′-factors were also calculated (Zhang et al., 1999). The Z′-factor was designed to reflect both assay signal-to-noise ratio and the variation associated with the signal measurements. The value can be used to evaluate the quality and reproducibility of high-throughput screening assays. In an ideal assay, the Z′-factor is close to 1.0. In practical terms, a Z′-factor greater than 0.5 is indicative of an excellent assay, whereas assays with Z′-factor values of less than 0.5 show a small separation band. The generation of singlet oxygen and superoxide from methotrexate (**36**, 200 μM) and erythromycin (**68**, 200 μM) under 1-h exposure to simulated sunlight was monitored 20 times (Fig. 1). The Z′-factors for singlet oxygen and superoxide generation were estimated to be 0.58 and 0.95, respectively, demonstrating that the micellar ROS assay had wide separation bands between positive and negative controls. From these findings, the mROS assay would have sufficient assay precisions and robustness for evaluating the photoreactivity of chemicals. However, the values of ROS generation from irradiated chemicals tended to be variable in the presence of 0.5% (v/v) Tween 20 and, in particular, the transition in superoxide value for methotrexate (**36**) was significant. These findings suggested that compartmentalization within micelles might cause changes in the photochemical behavior and ROS-generating properties of the tested chemicals, and/or redox behavior of the dye reagents. Further accumulation of ROS and mROS data would be required for precise photosafety evaluation.

3.3. Applicability to poorly soluble chemicals

Both ROS and mROS assays were carried out on 65 phototoxic compounds (**1–65**) and 18 non-phototoxic compounds (**66–83**) for comparative purposes, and the obtained results are summarized in Table 2. In the ROS assay, owing to limited aqueous solubility, 23 chemicals (27.7% of the total) could not be dissolved at a final concentration of 200 μM in the assay buffer. In contrast, the assay buffer for mROS assay could dissolve 81 chemicals (97.6% of the total), and anthracene (**62**) and UV-571 (**83**) were still poorly soluble even in the micellar solutions. From these findings,

Table 1
Intra-day and inter-day precisions of ROS and mROS assays.

Compounds (200 μM)	ROS assay		mROS assay	
	$^1O_2$ ($\Delta A_{440\,nm}\cdot 10^3$)[a]	O_2 ($\Delta A_{560\,nm}\cdot 10^3$)[b]	$^1O_2$ ($\Delta A_{440\,nm}\cdot 10^3$)[a]	O_2 ($\Delta A_{560\,nm}\cdot 10^3$)[b]
Intra-day (n = 9)				
Methotrexate	214 ± 20 (9.4)	320 ± 11 (3.6)	226 ± 22 (9.9)	927 ± 23 (2.5)
Erythromycin	−0.6 ± 3.2	−4.8 ± 12.9	2.1 ± 1.1	5.1 ± 4.7
Inter-day (n = 18)				
Methotrexate	206 ± 22 (10.8)	304 ± 24 (7.9)	224 ± 24 (10.9)	916 ± 24 (2.6)
Erythromycin	−1.3 ± 8.0	−2.1 ± 10.5	2.9 ± 1.5	5.9 ± 4.4

Compounds (200 μM) were dissolved in 20 mM NaPB (pH 7.4) with or without 0.5% (v/v) Tween 20 and exposed to simulated sunlight (2.0 mW/cm^2) for 1 h. Data represent mean ± SD of three repeated experiments for intra-day precision and six repeated experiments for intra-day experiments (days 1 and 2). Values in parentheses are coefficients of variation (CV,%).

[a] Decrease in $A_{440\,nm} \times 10^3$.

[b] Increase in $A_{560\,nm} \times 10^3$.

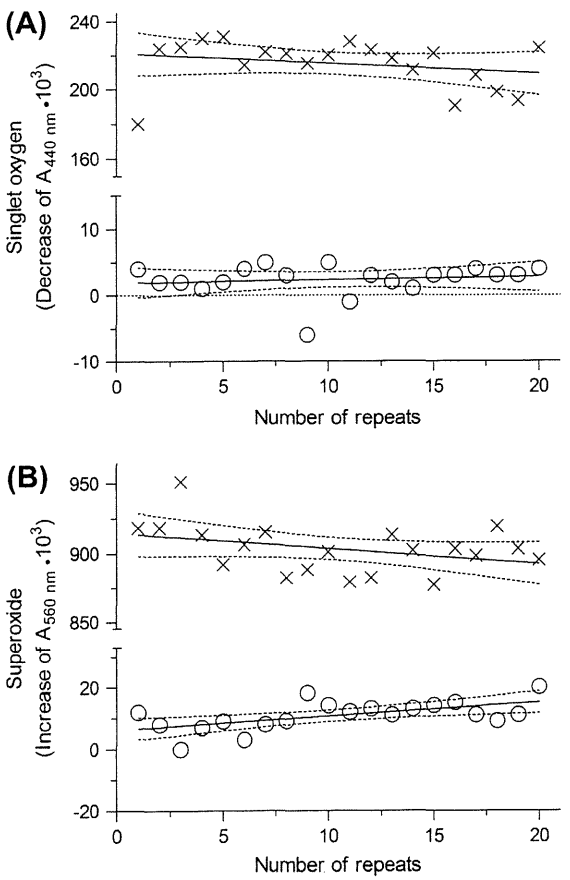

Fig. 1. Representative multiple measurement of singlet oxygen (A) and superoxide (B) to calculate the *Z'*-factor for the mROS assay. ×, Methotrexate (**36**) at 200 μM; and ○, erythromycin (**68**) at 200 μM. Solid and dashed lines represent mean and 95% confidence interval, respectively.

owing to the intense solubilization potency of Tween 20, the mROS assay achieved a marked increase in the number of evaluable compounds compared with the ROS assay.

In recent years, as much as ca. 70% of new drug candidates have shown poor aqueous solubility, and ca. 40% of marketed drugs for oral use are identified to be practically insoluble in aqueous media (<100 μg/mL) (Takagi et al., 2006). Thus, there are serious problems arising from the limited solubility of some new drug candidates in safety evaluation. The present observations suggest that the use of 0.5% (v/v) Tween 20 micellar solution for mROS assay led to successful solubilization of most poorly water-soluble chemicals, and thus the mROS assay might be a viable screening option for the photosafety assessment of poorly water-soluble drug candidates.

3.4. Prediction capacity and assay limitations

The photoreactivity of tested compounds was categorized by criteria of ROS generation proposed previously (Onoue et al., 2008a): (i) 25 ($\Delta A_{440\,nm}\cdot10^3$) for singlet oxygen, and (ii) 20 ($\Delta A_{560\,nm}\cdot10^3$) for superoxide. Thus, in Fig. 2, compounds in the shaded region appear to have a low potential for phototoxic skin responses. In the ROS assay, generation of superoxide from 9 chemicals, including benzbromarone (**8**), candesartan cilexetil (**11**), fluvastatin Na (**21**), meloxicam (**35**), piroxicam (**49**), rosiglitazone (**55**), avobenzone (**63**), bithionol (**64**) and SDS (**82**), were unevaluable because of poor solubility in the assay buffer. However, the generation of singlet oxygen from these chemicals could be measured, and the values of these chemicals except for meloxicam (**35**) and SDS (**82**) exceeded the criterion for singlet oxygen (Fig. 2A). Therefore, the 7 phototoxins were judged to be photoreactive. The mROS assay could monitor the generation of both singlet oxygen and superoxide from 80 tested compounds (Fig. 2B). Although superoxide data for glibenclamide (**23**) could not be obtained, it was found to be a potent singlet oxygen generator, and was thereby identified as photoreactive. In contrast, negative predictions could not be made for tested chemicals lying in the black region since either or both of ROS determinations were unavailable with sub-threshold level of measurable ROS. Herein, there appeared to be 25 and 2 unevaluable chemicals in the ROS and mROS assays, respectively.

The results of the ROS and mROS assays on evaluable compounds were compared with their photosafety information (Fig. 3A). In the ROS assay, 55 evaluable chemicals were consistent with the photosafety information, and only 3 compounds, including penicillin G (**69**), cinnamic acid (**74**) and L-histidine (**76**), were determined to be false positives. The mROS assay demonstrated that the ROS data on 75 evaluable compounds were in agreement with their *in vitro/in vivo* phototoxic information; however, there appeared to be 6 false predictions, including ibuprofen (**27**), indomethacin (**28**), penicillin G (**69**), phenytoin (**70**), cinnamic acid (**74**) and L-histidine (**76**). Individual specificities for the ROS and mROS assays were 70.0% and 76.5%, respectively. The positive and negative predictivities were found to be 94.1/100% for the ROS assay and 93.9/86.7% for the mROS assay. Lower classification criteria were set in the ROS assay to avoid false negative predictions, and the ROS assay sometimes provided false positive predictions. In the previous validation study, false positive predictions were also made for penicillin G (**69**), phenytoin (**70**), cinnamic acid (**74**) and L-histidine (**76**), and these observations were partly consistent with the photodegradation and photochemical reactions of these chemicals that occurred via radical reactions and/or electron transfer (Onoue et al., in press). Most notably, there appeared to be false negative predictions on ibuprofen (**27**) and indomethacin (**28**) in the mROS assay, whereas they were correctly determined to be positive in the ROS assay. The false negative predictions would imply the limitation of the mROS assay for photosafety assessment.

3.5. Proposed photosafety evaluation approach employing ROS and mROS assays

Because of some false negative predictions in the mROS assay, the ROS assay might provide more reliable photosafety prediction compared with the mROS assay. Indeed, the mROS assay had the limitation in its predictivity performance, although the solubilizing potency of the micellar solution of 0.5% (v/v) Tween 20 would be attractive for the photosafety assessment of poorly water-soluble chemicals. Thus, it would be of value if the mROS assay could be used as an optional assay applied for unevaluable chemicals in the ROS assay.

In a trial, the combined screening strategy was applied to 83 chemicals tested in the present study (Fig. 3B). Even in the micellar solution, only 2 chemicals, namely, anthracene (**62**) and UV-571 (**83**), were still poorly soluble, and thus they were subjected to the screening system at much lower concentrations: anthracene (**62**) at 50 μM and UV-571 (**83**) at 100 μM. According to the mROS data on anthracene {**62**, 50 μM: singlet oxygen ($\Delta A_{440\,nm}\cdot10^3$), 143 ± 2; and superoxide ($\Delta A_{560\,nm}\cdot10^3$), 344 ± 23} and UV-571 {**83**, 100 μM: singlet oxygen ($\Delta A_{440\,nm}\cdot10^3$), 3 ± 1; and superoxide ($\Delta A_{560\,nm}\cdot10^3$), <0.1}, anthracene (**62**) could be identified to be photoreactive; however, UV-571 (**83**) must be unevaluable in the current screening system. On the basis of combined use of the ROS and mROS assays, photosafety prediction on 82 chemicals

Table 2
ROS data on tested compounds.

No.	Compounds	CAS No.	ROS generation						Sources of photosafety information
			ROS assay		mROS assay[c]				
			$^1O_2$ ($\Delta A_{440\,nm} \cdot 10^3$) [a]	O_2^- ($\Delta A_{560\,nm} \cdot 10^3$) [b]	$^1O_2$ ($\Delta A_{440\,nm} \cdot 10^3$) [a]		O_2^- ($\Delta A_{560\,nm} \cdot 10^3$) [b]		
Phototoxic drugs (61)									
1	Acridine	260-94-6	217 ± 17	184 ± 18	255 ± 8	[38]	480 ± 9	[296]	Peters and Holzhutter (2002) and Spielmann et al. (1998b)
2	Acridine HCl	17784-47-3	196 ± 12	192 ± 5	250 ± 14	[54]	457 ± 11	[265]	Spielmann et al. (1998b) and Spielmann et al. (1998c)
3	Amiodarone HCl	19774-82-4	N.A.	N.A.	259 ± 9		369 ± 18		Moore (2002) and Spielmann et al. (1998a)
4	Amlodipine	88150-42-9	N.A.	N.A.	4 ± 2		631 ± 18		Grabczynska and Cowley (2000)
5	Amlodipine besylate	111470-99-6	11 ± 4	77 ± 5	2 ± 1	[−9]	607 ± 26	[530]	Grabczynska and Cowley (2000)
6	Atorvastatin	134523-00-5	N.A.	N.A.	846 ± 9		844 ± 5		Package insert
7	Benidipine HCl	91599-74-5	N.A.	N.A.	26 ± 1		924 ± 3		Package insert
8	Benzbromarone	3562-84-3	64 ± 10	N.A.	67 ± 8	[3]	N.D.		Package insert
9	Bezafibrate	41859-67-0	N.A.	40 ± 4	3 ± 2		132 ± 5	[92]	Canudas et al. (1996)
10	Bufexamac	2438-72-4	31 ± 3	N.D.	31 ± 1	[0]	N.D.	[0]	Kurumaji (1998)
11	Candesartan cilexetil	145040-37-5	33 ± 8	N.A.	100 ± 3	[67]	880 ± 13		Package insert
12	Chlorpromazine HCl	69-09-0	14 ± 3	90 ± 1	48 ± 3	[34]	60 ± 8	[−30]	Onoue et al. (2010) and Spielmann et al. (1998b)
13	Cilnidipine	132203-70-4	N.A.	N.A.	2 ± 1		891 ± 8		Package insert
14	Ciprofloxacin	85721-33-1	314 ± 16	46 ± 11	241 ± 3	[−73]	836 ± 50	[790]	Lipsky and Baker (1999) and Moore (2002)
15	Clofibrate	637-07-0	87 ± 7	N.D.	181 ± 3	[94]	N.D.	[0]	Moore (2002)
16	Diclofenac Na	15307-79-6	395 ± 1	420 ± 11	451 ± 5	[56]	N.D.	[−420]	Moore (2002)
17	Doxycycline HCl	10592-13-9	201 ± 10	329 ± 7	114 ± 9	[−87]	575 ± 2	[246]	Moore (2002) and Spielmann et al. (1994)
18	Enoxacin	74011-58-8	489 ± 20	224 ± 16	454 ± 19	[−35]	852 ± 8	[628]	Lipsky and Baker (1999) and Moore (2002)
19	Fenofibrate	49562-28-9	N.A.	N.A.	488 ± 6		208 ± 4		Peters and Holzhutter (2002) and Spielmann et al. (1998b)
20	Fluphenazine 2HCl	146-56-5	570 ± 11	49 ± 4	406 ± 11	[−164]	377 ± 3	[328]	Miolo et al. (2006)
21	Fluvastatin Na	93957-55-2	282 ± 6	N.A.	376 ± 15	[94]	313 ± 5		Viola et al. (2010)
22	Furosemide	54-31-9	187 ± 6	86 ± 3	245 ± 8	[58]	131 ± 10	[45]	Moore (2002) and Spielmann et al. (1998b)
23	Glibenclamide	10238-21-8	N.A.	N.A.	89 ± 5		N.A.		Moore (2002)
24	Gliclazide	21187-98-4	45 ± 3	218 ± 14	36 ± 1	[−9]	787 ± 6	[569]	Package insert
25	Griseofulvin	126-07-8	58 ± 10	12 ± 4	69 ± 5	[11]	165 ± 5	[153]	Moore (2002)
26	Hydrochlorothiazide	58-93-5	77 ± 4	N.D.	310 ± 6	[233]	N.D.	[0]	Moore (2002)
27	Ibuprofen	15687-27-1	5 ± 2	125 ± 1	11 ± 0	[6]	N.D.	[−125]	Moore (2002)
28	Indomethacin	53-86-1	14 ± 2	214 ± 7	23 ± 2	[9]	N.D.	[−214]	Moore (2002)
29	Ketoprofen	22071-15-4	310 ± 11	103 ± 4	568 ± 4	[258]	619 ± 10	[516]	Moore (2002) and Spielmann et al. (1998b)
30	Levofloxacin	100986-85-4	204 ± 9	372 ± 17	194 ± 12	[−10]	951 ± 4	[579]	Hayashi et al. (2004) and Lipsky and Baker (1999)
31	Lomefloxacin HCl	98079-52-8	689 ± 9	86 ± 11	593 ± 7	[−96]	786 ± 8	[700]	Hayashi et al. (2004) and Moore (2002)
32	Losartan K	124750-99-8	N.A.	N.A.	3 ± 3		732 ± 5		Package insert
33	Lovastatin	75330-75-5	N.A.	N.A.	63 ± 1		7 ± 1		Quiec et al. (1995)
34	Manidipine HCl	89226-75-5	N.A.	N.A.	156 ± 3		923 ± 6		Package insert
35	Meloxicam	71125-38-7	13 ± 2	N.A.	55 ± 2	[42]	223 ± 5		Package insert
36	Methotrexate	59-05-2	210 ± 20	325 ± 10	224 ± 29	[14]	918 ± 8	[593]	Moore (2002)
37	8-Methoxypsoralen	298-81-7	87 ± 10	38 ± 10	34 ± 2	[−53]	85 ± 4	[47]	Onoue et al. (2010) and Spielmann et al. (1994)
38	6-Methylcoumarin	92-48-8	107 ± 40	99 ± 3	76 ± 3	[−31]	67 ± 14	[−32]	Peters and Holzhutter (2002) and Spielmann et al. (1998b)
39	Mequitazine	29216-28-2	206 ± 5	62 ± 4	246 ± 15	[40]	114 ± 5	[52]	Fujita and Matsuo (1986)
40	Nalidixic acid	389-08-2	191 ± 12	195 ± 22	246 ± 14	[55]	634 ± 10	[439]	Moore (2002) and Peters and Holzhutter (2002)
41	Naproxen	22204-53-1	258 ± 13	391 ± 7	207 ± 7	[−51]	257 ± 9	[−134]	Moore (2002)
42	Nicardipine HCl	54527-84-3	N.A.	N.A.	187 ± 4		943 ± 8		Package insert
43	Nitrendipine	39562-70-4	N.A.	N.A.	9 ± 2		889 ± 4		Package insert
44	Norfloxacin	70458-96-7	258 ± 15	90 ± 13	162 ± 5	[−96]	933 ± 3	[843]	Peters and Holzhutter (2002) and Spielmann et al. (1998b)
45	Ofloxacin	82419-36-1	217 ± 13	367 ± 25	190 ± 14	[−27]	929 ± 3	[562]	Moore (2002) and Spielmann et al. (1998b)
46	Omeprazole	73590-58-6	N.A.	N.A.	297 ± 6		51 ± 2		Gebhardt et al. (2012)
47	Perphenazine	58-39-9	N.D.	187 ± 6	111 ± 5	[111]	N.D.	[−187]	Miolo et al. (2006) and Moore (2002)
48	Pirfenidone	53179-13-8	41 ± 1	89 ± 4	35 ± 1	[−6]	116 ± 11	[27]	Taniguchi et al. (2010)
49	Piroxicam	36322-90-4	210 ± 14	N.A.	325 ± 27	[115]	769 ± 15		Moore (2002) and Spielmann et al. (1995)
50	Pitavastatin Ca	147526-32-7	N.A.	N.A.	786 ± 7		108 ± 5		Viola et al. (2012)

51	Pravastatin Na	81131-70-6	N.D.	26 ± 9	9 ± 2	[9]	62 ± 7	[36]	Package insert
52	Prochlorperazine dimaleate	84-02-6	N.D.	121 ± 6	87 ± 5	[87]	N.D.	[−121]	Moore (2002)
53	Promethazine HCl	58-33-3	76 ± 2	74 ± 2	83 ± 4	[7]	N.D.	[−74]	Onoue et al. (2010) and Spielmann et al. (1995)
54	Quinine HCl	6119-47-7	489 ± 16	304 ± 3	446 ± 21	[−43]	862 ± 6	[558]	Moore (2002) and Onoue et al. (2010)
55	Rosiglitazone	122320-73-4	104 ± 4	N.A.	70 ± 5	[−34]	127 ± 11		Onoue et al. (in press)
56	Sparfloxacin	110871-86-8	60 ± 2	8 ± 5	21 ± 8	[−39]	42 ± 3	[34]	Hayashi et al. (2004) and Lipsky and Baker (1999)
57	Tamoxifen	10540-29-1	N.A.	N.A.	172 ± 5		994 ± 8		Interview form
58	Tetracycline HCl	64-75-5	158 ± 15	198 ± 4	85 ± 19	[−73]	921 ± 6	[723]	Moore (2002) and Spielmann et al. (1994)
59	Thioridazine HCl	130-61-0	133 ± 3	72 ± 2	172 ± 10	[39]	26 ± 6	[−46]	Miolo et al. (2006)
60	Trifluoperazine	440-17-5	632 ± 10	27 ± 2	432 ± 10	[−200]	228 ± 12	[201]	Moore (2002)
61	Valsartan	137862-53-4	16 ± 2	259 ± 28	18 ± 1	[2]	401 ± 4	[142]	Package insert
Phototoxic chemicals (4)									
62	Anthracene	120-12-7	N.A.	N.A.	N.A.		N.A.		Spielmann et al. (1998b) and Spielmann et al. (1998c)
63	Avobenzone	70356-09-1	142 ± 4	N.A.	57 ± 6	[−85]	N.D.		Motley and Reynolds (1989)
64	Bithionol	97-18-7	81 ± 3	N.A.	138 ± 8	[57]	N.D.		Spielmann et al. (1998c) and Spielmann et al. (1995)
65	Hexachlorophene	70-30-4	302 ± 3	21 ± 17	46 ± 2	[−256]	N.D.	[−21]	Durbize et al. (2003) and Spielmann et al. (1998b)
Non-phototoxic drugs (6)									
66	Aspirin	50-78-2	3 ± 2	N.D.	1 ± 1	[−2]	N.D.	[0]	Onoue et al. (2010)
67	Benzocaine	94-09-7	4 ± 1	9 ± 1	4 ± 1	[0]	N.D.	[−9]	Onoue et al. (2010)
68	Erythromycin	114-07-8	N.D.	N.D.	4 ± 1	[4]	12 ± 1	[12]	Onoue et al. (2010)
69	Peniciline G	113-98-4	N.D.	54 ± 8	10 ± 2	[10]	40 ± 4	[−14]	Spielmann et al. (1994) and Spielmann et al. (1995)
70	Phenytoin	57-41-0	1 ± 1	N.D.	13 ± 1	[12]	20 ± 1	[20]	Onoue et al. (2010)
71	Sulisobenzone	4065-45-6	N.D.	N.D.	N.D.	[0]	10 ± 1	[10]	Onoue et al. (2010) and Portes et al. (2002)
Non-phototoxic chemicals (12)									
72	Bumetrizole	3896-11-5	N.A.	N.A.	N.D.		N.D.		Onoue et al. (in press)
73	Chlorhexidine	55-56-1	N.D.	13 ± 6	20 ± 4	[20]	N.D.	[−13]	Peters and Holzhutter (2002) and Spielmann et al. (1994)
74	Cinnamic acid	140-10-3	N.D.	46 ± 22	6 ± 2	[6]	69 ± 2	[23]	Spielmann et al. (1995)
75	Drometrizole	2440-22-4	N.A.	N.A.	N.D.		N.D.		Onoue et al. (in press)
76	L-Histidine	71-00-1	N.D.	49 + 11	8 ± 3	[8]	35 ± 3	[−14]	Spielmann et al. (1994, 1995)
77	4-Methylbenzylidene camphor	36861-47-9	N.A.	N.A.	4 ± 1		N.D.		Spielmann et al. (1998c)
78	Octrizole	3147-75-9	N.A.	N.A.	N.D.		N.D.		Onoue et al. (in press)
79	Octyl methoxycinnamate	5466-77-3	N.A.	N.A.	N.D.		N.D.		Onoue et al. (in press)
80	Octyl salicylate	118-60-5	N.A.	N.A.	N.D.		N.D.		Spielmann et al. (1998c)
81	PABA	150-13-0	N.D.	N.D.	8 ± 2	[8]	N.D.	[0]	Peters and Holzhutter (2002) and Spielmann et al. (1995)
82	SDS	151-21-3	17 ± 3	N.A.	6 ± 2	[−11]	N.D.		Spielmann et al. (1998b,c)
83	UV-571	125304-04-3	N.A.	N.A.	N.A.		N.A.		Onoue et al. (in press)

The ROS assay and the mROS assay were carried out for tested chemicals (200 μM). Data represent mean ± SD of three experiments. N.D.: not detected; N.A.: not available due to limited solubility.

[a] Decrease in $A_{440\,nm} \times 10^3$.

[b] Increase in $A_{560\,nm} \times 10^3$.

[c] Numbers in box brackets indicate changes in ROS data from the ROS assay when 'N.D.' is treated as zero.

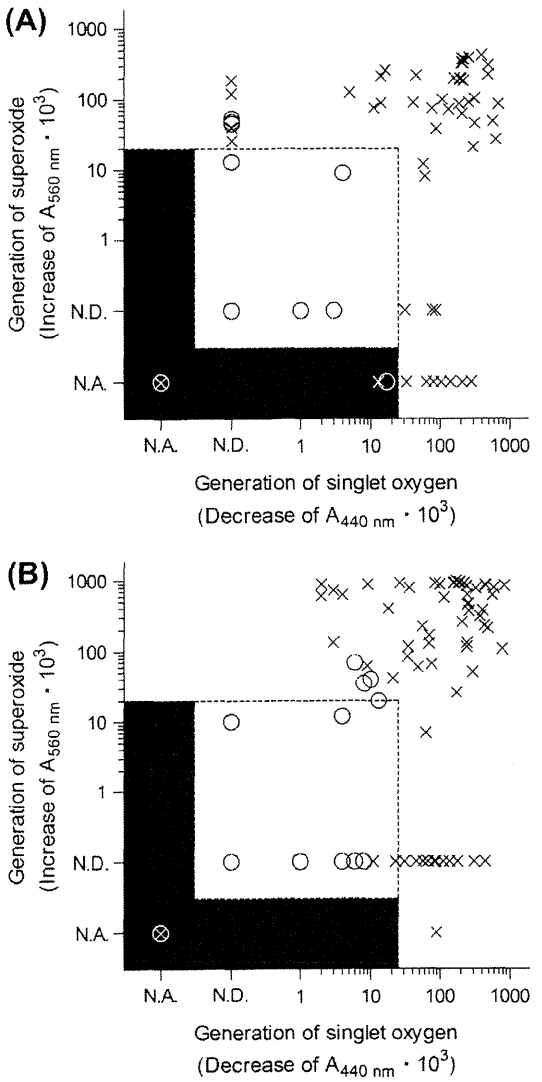

Fig. 2. 2D plots of singlet oxygen data *versus* superoxide data for 83 compounds (200 μM) obtained from the ROS assay (A) and the mROS assay (B). Data represent mean of three experiments. ○: Non-phototoxic drugs/chemicals; and ×: phototoxic drugs/chemicals. N.D.: Not detected; and N.A.: not available due to limited solubility. According to the criteria for phototoxins (200 μM) defined previously (Onoue et al., 2008a), the gray and white regions are indicative of less photoreactive and photoreactive, respectively. The black region indicates that compounds could not be rated due to their poor solubility.

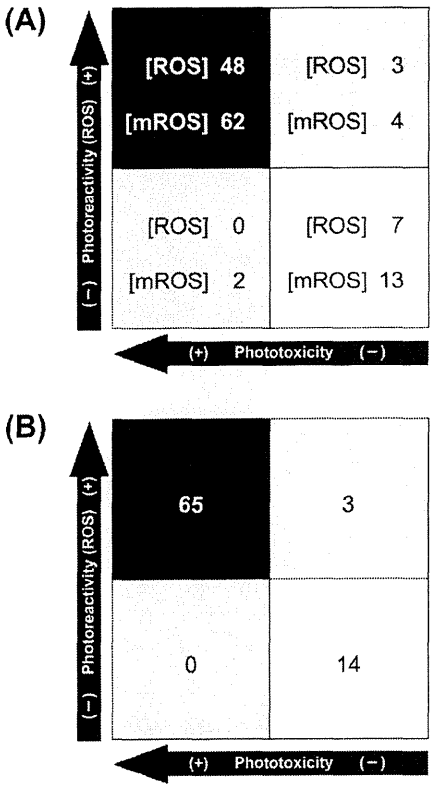

Fig. 3. Prediction capacity of ROS or mROS assay (A) and the combined use of these two assays (B) for the *in vitro/in vivo* photosafety of tested chemicals.

(98.8% of the total) could be made with individual specificity, positive and negative predictivities of 82.4%, 95.6% and 100%, respectively. From these findings, the proposed screening strategy demonstrated high applicability and prediction performance. Thus, a high-throughput screening strategy upon combined use of the ROS and mROS assays could be proposed for reliable photosafety assessment.

In this proposed strategy (Fig. 4), the ROS assay should be firstly employed for risk assessment, and chemicals tested at 200 μM can be identified to be photoreactive or not on the basis of tentative classification criteria. As observed in the present study, the ROS assay would sometimes be unavailable due to poor solubility of the tested chemicals. According to the validated protocol for the ROS assay (Onoue et al., in press), when precipitation could be observed in assay buffer containing the tested chemical at 200 μM under an optical microscope, additional experiments could be performed at lower concentration. The threshold values 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. However, the tested chemicals could also be identified to be phototoxic as long as the ROS data at lower concentrations surpassed these 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 the ROS assay under dilution. Herein, as well as insoluble chemicals, the diluted samples with a subthreshold level of ROS should be identified to be unevaluable in the ROS assay. The mROS assay can then be applied to these imponderable chemicals for follow-up screening. Empirically, this would enable photoreactivity assessment of most chemicals at 200 μM or under appropriate dilution, whereas careful consideration should also be made on negative predictivity for the diluted samples. On the basis of present outcomes, the complementary use of mROS assay would be of great help in photosafety assessment on poorly water-soluble chemicals in early stages of drug discovery and product development.

4. Conclusion

The mROS assay was developed with the use of 0.5% (v/v) Tween 20 for photosafety assessment on poorly water-soluble chemicals. This mROS assay exhibited high robustness and reproducibility, and the addition of micellar solution significantly enhanced the applicability of the ROS assay system to poorly water-soluble chemicals. Despite some false negative predictions in the mROS assay, complementary use of mROS assay might strengthen the assay performance of the ROS assay on wide range of new drug candidates for exploratory and regulatory purposes.

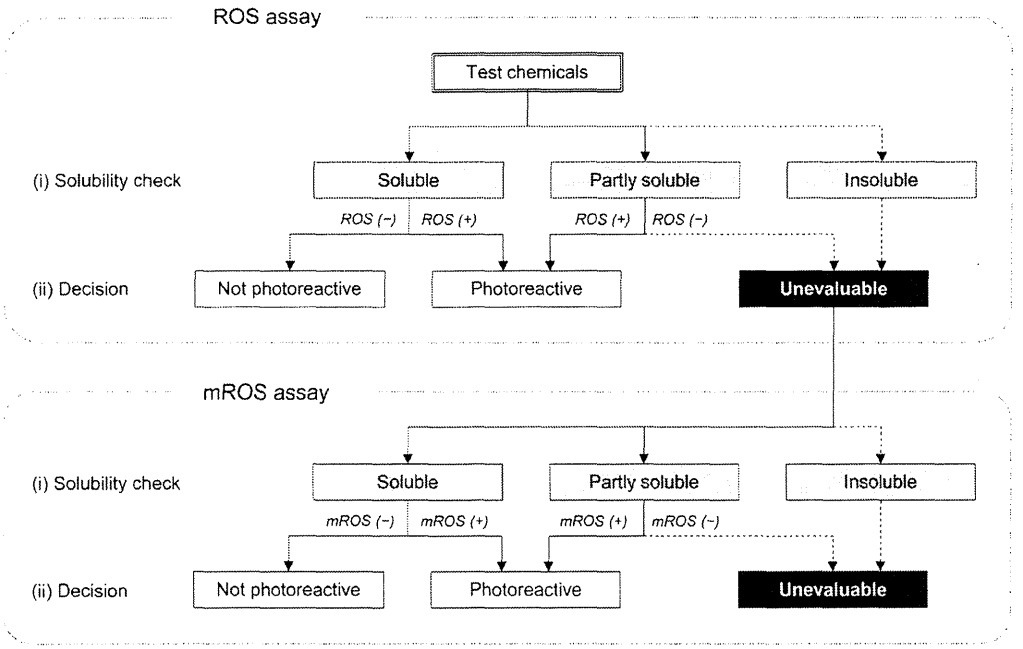

Fig. 4. Schematic representation of proposed photosafety evaluation approach with combined use of ROS and mROS assays. Upon solubility check, each tested chemical was divided into 3 groups as follows; (1) Soluble: Chemical (200 μM) is soluble in both assay mixtures for determination of singlet oxygen and superoxide; (2) Partly soluble: (i) Chemical (200 μM) is insoluble in either assay buffer, or (ii) Diluted chemical (<200 μM) is soluble in at least one of assay buffers; and (3) Insoluble: Chemical is insoluble in both assay buffers.

Conflict of Interest

None of the authors have any conflicts of interest associated with this study.

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Non-animal photosafety assessment approaches for cosmetics based on the photochemical and photobiochemical properties

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ABSTRACT

The main purpose of the present study was to establish a non-animal photosafety assessment approach for cosmetics using *in vitro* photochemical and photobiochemical screening systems. Fifty-one cosmetics, pharmaceutics and other chemicals were selected as model chemicals on the basis of animal and/or clinical photosafety information. The model chemicals were assessed in terms of photochemical properties by UV/VIS spectral analysis, reactive oxygen species (ROS) assay and 3T3 neutral red uptake phototoxicity testing (3T3 NRU PT). Most phototoxins exhibited potent UV/VIS absorption with molar extinction coefficients of over 1000 $M^{-1}cm^{-1}$, although false-negative prediction occurred for 2 cosmetic phototoxins owing to weak UV/VIS absorption. Among all the cosmetic ingredients, ca. 42% of tested chemicals were non-testable in the ROS assay because of low water solubility; thereby, micellar ROS (mROS) assay using a solubilizing surfactant was employed for follow-up screening. Upon combination use of ROS and mROS assays, the individual specificity was 88.2%, and the positive and negative predictivities were estimated to be 94.4% and 100%, respectively. In the 3T3 NRU PT, 3 cosmetics and 4 drugs were incorrectly predicted not to be phototoxic, although some of them were typical photoallergens. Thus, these *in vitro* screening systems individually provide false predictions; however, a systematic tiered approach using these assays could provide reliable photosafety assessment without any false-negatives. The combined use of *in vitro* assays might enable simple and fast non-animal photosafety evaluation of cosmetic ingredients.

1. Introduction

Exogenous phototoxicity can be defined as a toxic reaction in light-exposed tissues such as skin and eyes, elicited by topical or systemic application of chemicals and subsequent exposure to sunlight or artificial light (Moore, 2002). Phototoxicity is of increasing concern in dermatology because of the increased level of ultraviolet (UV) radiation from the sun reaching the earth (Onoue et al., 2009). Phototoxicity can be caused by several classes of pharmaceutics, cosmetics and food (Moore, 1998, 2002), which have the potential to provoke photoirritant, photoallergic and photogenotoxic events in light-exposed tissues through oxidation, chemical modification and covalent binding with endogenous biomolecules under exposure to sunlight (Epstein, 1983). Cosmetic products were often designed in the desire for retention of the agent on the skin despite rinsing, and the repeated application of phototoxic cosmetics to the skin would necessarily increase risk. Previously, owing to numerous reports on their photoallergic potential, cosmetic use of 6-methylcoumarin, musk ambrette and hexachlorophene has declined significantly (Allen et al., 1997). With the aim of reducing and preventing cosmetic phototoxicity, increasing attention has been drawn to photosafety assessment of cosmetic ingredients and products.

Photosafety assessments of cosmetics have been generally carried out by *in vitro* and *in vivo* phototoxicity testing, although the safety testing approaches are currently undergoing a drastic paradigm shift due to regulatory requirements. The 7th Amendment (Directive 2003/15/EC) to the Cosmetic Directive (Directive76/768/EEC) calls for a marketing ban, from March

Abbreviations: 3T3 NRU PT, 3T3 neutral red uptake phototoxicity testing; ANOVA, one-way analysis of variance; CMC, critical micelle concentration; DMSO, dimethyl sulfoxide; ECVAM, European Centre for the Validation of Alternative Methods; ICH, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; IFRA, International Fragrance Association; JaCVAM, Japanese Center for the Validation of Alternative Methods; JPMA, Japan Pharmaceutical Manufacturers Association; MEC, molar extinction coefficient; mROS assay, micellar ROS assay; NaPB, sodium phosphate buffer; NBT, nitroblue tetrazolium; OECD, Organisation for Economic Co-operation and Development; photo-h-CLAT, photo human cell line activation test; PIF, photoirritation factor; ROS, reactive oxygen species; UV, ultraviolet; VIS light, visible light.

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2013, on cosmetic products that contain ingredients tested on animals for toxicity and toxicokinetics (Adler et al., 2011). This directive is intended to protect and improve the welfare of animals for experimental and research purposes by promoting the alternative use of established *in vitro* testing systems (Balls and Clothier, 2010). In addition, regulatory agencies have recommended the implementation of the 3Rs principle (refinement, reduction, replacement), so interest in the development of non-animal photosafety assessments is increasing rapidly (Seto et al., 2012). Therefore, instead of *in vivo* hazard characterization of new cosmetic ingredients, strategic development of *in vitro*, *in chemico* and/or *in silico* models has become a high priority for the replacement of animal testing (Maxwell et al., 2011).

A number of effective *in vitro* methodologies have been developed for photosafety assessment of pharmaceutical substances over the past few years, and guidance on the photosafety testing of medicinal products was established by regulatory agencies in the US and EU in the early 2000s (Seto et al., 2012). Recently, the issuance of the Step 2 draft ICH S10 photosafety guidance document provided a detailed framework and guidance for photosafety evaluation (ICH, 2013). These guidelines describe photosafety assessment strategies on the basis of photochemical and photobiochemical properties, and *in vivo* pharmacokinetic behavior (EMEA/CPMP, 2002; FDA/CDER, 2002; OECD, 2004). Application of all the proposed assays to cosmetics would be challenging due to a full ban of *in vivo* studies on cosmetic ingredients and products. However, strategic use of some *in vitro* photochemical and photobiochemical methods might still provide reliable photosafety information on cosmetics, and they include UV spectral analysis for evaluating UV-absorbing properties (Henry et al., 2009), reactive oxygen species (ROS) assay for photoreactivity (Onoue and Tsuda, 2006) and 3T3 neutral red uptake phototoxicity test (3T3 NRU PT) for cytotoxicity of photoactivated chemicals (Spielmann et al., 1994). These photosafety screening systems are well validated with high predictive capacity for pharmaceutical substances; however, the applicability and predictive performance for cosmetics are still unclear.

The major purpose of the present study is to establish a photosafety testing strategy for cosmetics employing *in vitro* photochemical and photobiochemical data. Here, 34 phototoxins (20 cosmetics and 14 non-cosmetics) with photoirritant and/or photoallergic potential and 18 non-phototoxic chemicals (14 cosmetics and 4 non-cosmetics) were selected as model chemicals on the basis of reported photosafety information (Hoya et al., 2009; Lovell and Jones, 2000; Moore, 2002; Onoue et al., 2013; Onoue and Tsuda, 2006; SCCNFP, 1999, 2004; SCCP, 2006a,b, 2008; Tokura, 1998, 2009) and the International Fragrance Association (IFRA) standard. IFRA standard forms the basis for the globally accepted and recognized risk management system to ensure the safe use of fragrance ingredients. Photochemical properties of model chemicals were assessed by UV/VIS spectral analysis (Henry et al., 2009) and ROS assay (Onoue et al., 2013). Many cosmetics were nontestable in the ROS assay due to poor solubility in aqueous medium, so the micellar ROS (mROS) assay, originally developed for photosafety testing of lipophilic drugs, was also applied to these (Seto et al., 2013). *In vitro* photosafety testing of these chemicals was conducted using 3T3 NRU PT, and all *in vitro* photosafety data were integrated for strategic prediction of phototoxicity and the proposal of a new photosafety testing strategy for cosmetics.

2. Materials and methods

2.1. Chemicals

According to previous *in vitro*/*in vivo* photosafety information and clinical observations (Hoya et al., 2009; Lovell and Jones, 2000; Moore, 2002; Onoue et al., 2013; Onoue and Tsuda, 2006; SCCNFP, 1999, 2004; SCCP, 2006a,b, 2008; Tokura, 1998, 2009), 51 chemicals, including 33 cosmetics and 18 non-cosmetics, were selected as model chemicals for the present study (Table 1). Dimethyl sulfoxide (DMSO, **48**), *p*-nitrosodimethylaniline, imidazole, nitroblue tetrazolium (NBT), 3,4′,5-tribromosalicylanilide (**1**), 4-methyl-7-ethoxycoumarin (**2**), 7-methoxycoumarin (**5**), 8-methoxypsoralen (**6**), hexachlorophene (**11**), methyl β-naphthylketone (**12**), *p*-phenylenediamine (**18**), tetrachlorosalicylanilide (**19**), acridine (**21**), amiodarone HCl (**22**), chlorpromazine HCl (**24**), diclofenac Na (**25**), fenofibrate (**27**), indomethacin (**28**), ketoprofen (**29**), piroxicam (**30**), promethazine HCl (**31**), quinine HCl ($2H_2O$) (**32**), sulfanilamide (**33**), tetracycline HCl (**34**), 1,3-butylene glycol (**35**), 2-propanol (**36**), 4′-methylbenzylidene camphor (**37**), ethanol (**40**), glycerine (**41**) and isopropyl myristate (**42**) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Bithionol (**8**), dichlorophene (**9**), musk ketone (**15**), octyl dimethyl PABA (**17**), triclocarban (**20**), enoxacin (**26**), sulisobenzone (**47**), lactic acid (**49**) and penicillin G (**51**) were purchased from Sigma–Aldrich Japan (Tokyo, Japan). Benzophenone (**7**), lauric acid (**43**), propylene glycol (**44**), sodium lauryl sulfate (**46**) and methyl salicylate (**50**) were obtained from Junsei Chemical Co. (Tokyo, Japan), and 5-methoxypsoralen (**3**), 6-methylcoumarin (**4**), ascorbic acid (**38**), cetyl alcohol (**39**) and sodium laurate (**45**) were purchased from Nacalai Tesque (Kyoto, Japan). Fenticlor (**10**), methyl-*N*-methylanthranilate (**13**), musk ambrette (**14**) and musk xylene (**16**) were purchased from Tokyo Chemical Industry (Tokyo, Japan). A quartz reaction container for high-throughput ROS assay (Onoue et al., 2008a) was constructed by Ozawa Science (Aichi, Japan).

2.2. UV/VIS spectral analysis and determination of molar extinction coefficient (MEC)

Each chemical was dissolved in ethanol, methanol, acetone or distilled water at final concentrations of 0.001, 0.01 and 0.1 μM, and the final concentration was reduced if the tested chemical was found to be an intense UV/VIS absorber. UV/VIS absorption spectra were recorded with a UV–VIS Multipurpose Spectrophotometer MPS-2400 (Shimazu Corporation, Kyoto, Japan) interfaced to a PC for data processing (software: UV Prove Version 1.12). A spectrofluorimeter quartz cell with 10 mm pathlength was employed. MEC values were determined from absorbance values for peaks tailing through 290 nm from a previous maximum absorbance and all peaks detected at 290 nm and above.

2.3. ROS assay

2.3.1. Irradiation

The ROS assays were conducted using Atlas Suntest CPS plus (Atlas Material Technology LLC, Chicago, 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. The irradiation test was carried out at 25 °C with an irradiance of ca. 2.0 mW/cm^2 as determined by the calibrated UVA detector Dr. Hönle #0037 (Dr. Hönle AG UV Technology, München, Germany).

2.3.2. ROS assay

In accordance with the validated protocol with minor modification (Onoue et al., 2013), ROS assay was carried out to detect both singlet oxygen and superoxide generated from photo-irradiated chemicals. In the validated protocol, tested chemicals were diluted for the ROS assay if they were insoluble in assay solution. However, in the present study, ROS assay was conducted at the final concentration of 200 μM, and the insoluble chemicals were