



Comparative study on prediction performance of photosafety testing tools on photoallergens



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ABSTRACT

Several testing methods have been established to identify potential phototoxins. The present study was undertaken to clarify the predictive ability of *in vitro* photosafety assays for photoallergenicity. On the basis of animal and/or clinical photosafety information, 23 photoallergens and 7 non-phototoxic/non-photoallergenic chemicals were selected and subjected to UV/VIS spectral analysis, reactive oxygen species (ROS)/micellar ROS (mROS) assays, and 3T3 neutral red uptake phototoxicity testing (3T3 NRU PT). Of the photoallergens tested, ca. 96% of chemicals had intense UV/VIS absorption with a molar extinction coefficient of over $1000 \text{ M}^{-1} \text{ cm}^{-1}$, and false-positive predictions were made for 3 non-photoallergenic chemicals. In the ROS assay, all photoallergens were found to be potent ROS generators under exposure to simulated sunlight. In the photosafety prediction based on the ROS assay, the individual specificity was 85.7%, and the positive predictivity and negative predictivity were found to be 95.8% and 100%, respectively. Most of the photoirritant chemicals were correctly identified by the 3T3 NRU PT; however, it provided false predictions for ca. 48% of photoallergens. The orders of sensitivity and specificity for photoallergenicity prediction were estimated to be: [sensitivity] ROS assay > UV/VIS absorption > 3T3 NRU PT, and [specificity] 3T3 NRU PT > ROS assay > UV/VIS absorption. Thus, photochemical assays, in particular the ROS assay, can be used for assessment of photoallergenicity, although there were some false-positive predictions.

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1. Introduction

Phototoxic responses in light-exposed tissues can be caused by several classes of pharmaceuticals, cosmetics and foods, and these phototoxic events can be categorized into photoirritation, photoallergy and photogenotoxicity in accordance with their mechanisms and outcomes (Moore, 1998, 2002). Concerns about phototoxicity and its avoidance are growing, and a number of *in vitro* assay systems have been developed for photosafety assessment over the past few years (Onoue et al., 2009). The International Conference on Harmonization (ICH) S10 guidelines on photosafety evaluation

successfully reached step 5 of the ICH process in 2014, describing detailed photosafety assessment strategies on the basis of photochemical and photobiochemical properties, and *in vivo* pharmacokinetic behavior (ICH, 2014). In the ICH S10 guideline, three *in vitro* photosafety test methods are recommended: (i) UV spectral analysis (Henry et al., 2009), (ii) reactive oxygen species (ROS) assay (Onoue and Tsuda, 2006), and (iii) 3T3 neutral red uptake phototoxicity test (3T3 NRU PT) (Spielmann et al., 1994).

Absorption of sunlight by phototoxins, followed by photochemical reaction, is considered to be a key trigger for phototoxicity (Onoue et al., 2013b), because photo-excited chemicals may react with biomolecules, leading to phototoxic events (Moore, 1998, 2002). In this context, the UV-absorbing property of chemicals can be a potential indicator for phototoxic risk, and Henry and co-workers demonstrated that chemicals with a molar extinction coefficient (MEC) of less than $1000 \text{ M}^{-1} \text{ cm}^{-1}$ showed low phototoxic risk (Henry et al., 2009). Photo-excited chemicals tend to generate ROS, resulting in oxidative damage to the cellular membrane, DNA and other biomolecules (Brendler-Schwaab et al., 2004; Epstein and Wintroub, 1985); therefore, the ROS assay of photoirradiated chemicals has been used for photosafety assessment (Onoue et al., 2014). On the other hand, 3T3 NRU PT was originally established to assess the cytotoxicity of

Abbreviations: 3T3 NRU PT, 3T3 neutral red uptake phototoxicity testing; AOP, Adverse Outcome Pathway; DMSO, dimethyl sulfoxide; 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; PIF, photoirritation factor; ROS, reactive oxygen species; UV, ultraviolet; VIS light, visible light.

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photo-excited chemicals as an *in vitro* alternative to *in vivo* phototoxicity tests (Liebsch and Spielmann, 2002).

Photoirritation is a narrowly specified type of phototoxicity that can be defined as an inflammatory event in UV-exposed tissues, triggered by photo-oxidation of lipids and proteins in the cellular membrane (Girotti, 2001; Schothorst et al., 1972), while photoallergy is an immune response to photosensitizer-bound proteins (Tokura, 2009). The *in vitro* assays recommended in the ICH S10 guideline are well validated and have a high predictive capacity for photoirritancy of tested chemicals (ICH, 2014). Although a previous study demonstrated that the photoallergenic potential of tested chemicals might be partly identified by ROS assay (Onoue et al., 2013c), the applicability of these *in vitro* assessments for predicting photoallergenic risk is still poorly understood. Therefore, we undertook the present study to clarify the predictive performance of photochemical and photobiochemical assays for the photoallergenic potential, using a panel of 23 photoallergens and 7 non-phototoxic/non-photoallergenic chemicals (Table 1). These model chemicals were assessed by means of UV/VIS spectral analysis, ROS assay, and 3T3 NRU PT, and the results were compared.

2. Materials and methods

2.1. Chemicals

On the basis of published photosafety data and the International Fragrance Association (IFRA) standard (Bakkum and Heule, 2002; Hindsen et al., 2006; Horio et al., 1994; Kerr et al., 2010; Lovell, 1993; Lugovic et al., 2007; Moore, 2002; Murata et al., 1998; Onoue et al., 2013c; Scheinfeld et al., 2014; Tokura, 2009), 30 chemicals, including 23 photoallergens and 7 non-phototoxic chemicals, were selected for the present study (Table 1). Dimethyl sulfoxide (DMSO), erythromycin

(27), glibenclamide (8), hexachlorophene (9), indomethacin (11), imidazole, ketoprofen (13), 8-methoxypsoralen (2), 4'-methylbenzylidene camphor (24), nitroblue tetrazolium (NBT), *p*-nitrosodimethylaniline, phenytoin (30), piroxicam (18), sulfanilamide (20) and tribromsalan (22) were bought from Wako Pure Chemical Industries (Osaka, Japan). Aspirin (25), bithionol (4), dichlorophene (5), enoxacin (6), octyl dimethyl PABA (16), penicillin G (29), pyridoxine HCl (19) and triclocarban (23) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Benzophenone (3), hydrochlorothiazide (10) and methylsalicylate (28) were obtained from Junsei Chemical Co. (Tokyo, Japan), and 6-methylcoumarin (1) was purchased from Nacalai Tesque (Kyoto, Japan). Benzocaine (26), fenticlor (7), musk ambrette (14) and musk xylene (15) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Isoniazid (12) was obtained from LKT Laboratories (St. Paul, MN, USA). Omadine Na (17) was bought from Alfa Aesar (Heysham, UK). Sulfasalazine (21) was purchased from Fluka (St. Gallen, Switzerland). 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

Each chemical was dissolved in methanol or distilled water at final concentrations of 0.001, 0.01 and 0.1 μ M, 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 (Shimadzu Corporation, Kyoto, Japan) interfaced to a PC for data processing (software: UVProbe Version 1.12). MEC values were determined from absorbance values for peaks tailing through 290 nm from a previous maximum absorbance, and all peaks were detected at 290 nm or higher wavelength.

Table 1
Test chemicals.

No.	Chemical name	CAS number	Physical state	Color	Molecular weight	Clog P^b	Category ^c
<i>Photoallergens</i>							
1	6-Methylcoumarin	92-48-8	Powder	White	160.05	1.91	C
2	8-Methoxypsoralen	298-81-7	Powder	Light yellow	216.04	2.31	C, P
3	Benzophenone	119-61-9	Powder	White	182.07	3.18	C
4	Bithionol	97-18-7	Powder	White	353.88	6.16	C
5	Dichlorophene	97-23-4	Powder	Light yellow	268.01	4.79	P
6	Enoxacin	74011-58-8	Powder	White	320.13	−1.60	P
7	Fenticlor	97-24-5	Powder	Light yellow	285.96	5.19	C
8	Glibenclamide	10238-21-8	Powder	White	493.14	4.24	P
9	Hexachlorophene	70-30-4	Powder	White	403.85	7.03	C
10	Hydrochlorothiazide	58-93-5	Powder	White	296.96	−0.36	P
11	Indomethacin	53-86-1	Powder	Yellow	357.08	4.18	P
12	Isoniazid	54-85-3	Powder	White	137.06	−0.67	P
13	Ketoprofen	22071-15-4	Powder	White	254.09	2.76	P
14	Musk ambrette	83-66-9	Powder	Yellow	268.11	3.84	C
15	Musk xylene	81-15-2	Powder	White	297.10	3.96	C
16	Octyl dimethyl PABA	21245-02-3	Liquid	Light yellow	277.20	6.16	C
17	Omadine Na	3811-73-2	Powder	White	150.00 (127.01) ^a	−0.59	C
18	Piroxicam	36322-90-4	Powder	White	331.06	1.89	P
19	Pyridoxine HCl	58-56-0	Powder	White	205.05 (169.07) ^a	−0.35	P, F
20	Sulfanilamide	63-74-1	Powder	White	172.03	−0.57	P
21	Sulfasalazine	599-79-1	Powder	Yellow	398.07	3.99	P
22	Tribromsalan	87-10-5	Powder	White	446.81	6.01	P (animal)
23	Triclocarban	101-20-2	Powder	White	313.98	5.47	C
<i>Non-phototoxic/non-photoallergic chemicals</i>							
24	4'-Methylbenzylidene camphor	36861-47-9	Powder	White	254.17	5.02	C
25	Aspirin	50-78-2	Powder	White	180.04	1.02	P
26	Benzocaine	94-09-7	Powder	White	165.08	1.92	P
27	Erythromycin	114-07-8	Powder	White	791.47	1.66	P
28	Methylsalicylate	119-36-8	Liquid	Yellow	152.05	2.33	P
29	Penicillin G	113-98-4	Powder	White	387.08 (348.11) ^a	2.27	P
30	Phenytoin	57-41-0	Powder	White	252.09	2.09	P

^a Number in parenthesis represents molecular weight of free compound.

^b Calculated on ChemBioDraw Ultra 13.0 software.

^c C, cosmetic ingredients; F, food ingredients; and P, pharmaceuticals.

2.3. ROS assay

2.3.1. Irradiation

In the ROS assays, Atlas Suntest CPS plus (Atlas Material Technology LLC, Chicago, USA) was used as reported previously (Onoue et al., 2013a, 2014). The irradiation test was carried out at 25 °C with an irradiance of ca. 2.0 mW/cm².

2.3.2. ROS assay

The ROS assay was conducted in accordance with the validated protocol (Onoue et al., 2013a, 2014). Briefly, for the measurement of singlet oxygen, samples containing the test chemical (200 μM), *p*-nitrosodimethylaniline (50 μM) and imidazole (50 μM) in 20 mM NaPB (pH 7.4) were mixed in a tube. Two hundred microliters of the sample was transferred into a well of a plastic 96-well plate (Asahi Glass Co., Ltd., Tokyo, Japan; code number: 3881-096; clear, untreated, flat-bottomed) and checked for precipitation before light exposure. After measurement of absorbance at 440 nm using a SAFIRE microplate spectrophotometer (TECAN, Männedorf, Switzerland), the plate was fixed in a quartz reaction container with a quartz cover, then irradiated with simulated sunlight for 1 h, and agitated on a plate shaker. Then, the UV absorbance at 440 nm was measured. For the determination of superoxide, samples containing the test chemical (200 μM) and NBT (50 μM) in 20 mM NaPB were irradiated with simulated sunlight for 1 h, and the reduction in NBT was measured in terms of the increase in absorbance at 560 nm in the same manner as for the singlet oxygen determination.

2.3.3. mROS assay

The mROS assay was employed for chemicals that were untestable in the ROS assay because of limited solubility (Seto et al., 2013). Briefly, to monitor the generation of singlet oxygen, test compound (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, test compound (200 μM) and NBT (50 μM) were dissolved in 20 mM NaPB (pH 7.4) with 0.5% (v/v) Tween 20. These samples were then irradiated with simulated sunlight and measured under the same conditions as for the ROS assay protocol.

2.4. 3T3 NRU PT

The *in vitro* 3T3 NRU PT was carried out as described in the Organisation for Economic Co-operation and Development (OECD) 432 guidelines and the European Community Official Journal (L 136/9, 08.06.2000, annexe II) with minor modification (OECD, 2004). Briefly, Balb/c 3T3 cells were maintained in culture for 24 h for monolayer formation. Two 96-well plates per test chemical were then pre-incubated with six different concentrations of the chemical in duplicate for 1 h. One plate was exposed to a dose of 5 J/cm² UVA (+ Irr experiment), whereas the other plate was kept in the dark (− Irr experiment). UVA irradiation was performed using a SOL 500 Sun Simulator (Dr. Hönle AG UV Technology, München, Germany) equipped with a 500 W metal halide lamp and an H1 filter to attenuate UVB. The treatment medium was then replaced with culture medium. After 24 h, cell viability was determined by measuring neutral red uptake for 3 h; the neutral red uptake was measured at the absorbance of 540 nm using a Benchmark™ Plus microplate spectrophotometer (BioRad, Hercules, CA, USA). Cell viability at each of the six concentrations of the test chemical was compared with that of untreated controls and the percent inhibition was calculated. For prediction of the phototoxic potential, the concentration responses obtained in the presence and in the absence of UV irradiation were compared, usually at the IC₅₀ level, that is, the concentration that reduced cell viability to

50% of that of the untreated controls. The photoirritancy factor (PIF) was determined as follows:

$$\text{PIF} = \frac{\text{IC}_{50}(-\text{Irr})}{\text{IC}_{50}(+\text{Irr})}.$$

3. Results

3.1. UV/VIS spectral analysis

All the test chemicals were subjected to UV/VIS spectral analysis to evaluate photoreactivity (Table 2). Although weak UV absorption was seen for musk xylene (**15**), a weak photoallergen (SCCNFP, 2004), with an MEC value of 448 M^{−1} cm^{−1} (290 nm), most photoallergens exhibited potent UV/VIS absorption, with maximal MEC values of over 1000 M^{−1} cm^{−1}. In particular, the maximal MEC values of octyl dimethyl PABA (**16**) and sulfasalazine (**21**) within the UV/VIS region were found to be 28,879 M^{−1} cm^{−1} (310 nm) and 33,116 M^{−1} cm^{−1} (366 nm), respectively. Application of the threshold MEC value (1000 M^{−1} cm^{−1}) proposed by Henry and co-workers (Henry et al., 2009) correctly predicted ca. 96% of the chemicals to be photoallergenic (Table 3). Within the non-phototoxic/non-photoallergenic group, there appeared to be three false-positive predictions, since 4'-methylbenzylidene camphor (**24**), benzocaine (**26**) and methylsalicylate (**28**) exhibited intense UV absorption with MEC values of 11,876 M^{−1} cm^{−1} (299 nm), 17,025 M^{−1} cm^{−1} (290 nm), and 4722 M^{−1} cm^{−1} (308 nm), respectively. These observations are in agreement with previous reports that some non-phototoxic chemicals have intense UV/VIS absorption (Onoue and Tsuda, 2006), and the defined threshold (1000 M^{−1} cm^{−1}) might not always be reliable for negative prediction of photoallergenic potential. The positive and negative predictivities of the MEC-based approach were calculated to be 88.0% and 80.0%, respectively.

3.2. ROS assay

The ROS assay, originally developed for photosafety assessment of drugs, can monitor the generation of ROS such as singlet oxygen and superoxide from photoirradiated chemicals (Onoue et al., 2008b; Onoue and Tsuda, 2006). A validation study of the ROS assay was carried out by the Japan Pharmaceutical Manufacturers Association (JPMA), supervised by the Japanese Center for the Validation of Alternative Methods (JaCVAM) (Onoue et al., 2013a). The ROS assay was applied to all the test chemicals (Table 2); however, 8 chemicals (ca. 27% of total) were found to be untestable at the final concentration of 200 μM because of poor solubility. Therefore, mROS assay employing a micellar solution was optionally applied to these chemicals (Seto et al., 2013); this complementary use of mROS assay enabled us to conduct photosafety screening on 7 of the 8 chemicals (**5**, **7**, **14**–**16**, and **23**–**24**) previously untestable at 200 μM, and on tribromsalan (**22**) at a lower concentration (100 μM). In the ROS/mROS assays (Table 2), all photoallergens exhibited significant generation of ROS on exposure to simulated sunlight. Several photoallergens, such as enoxacin (**6**) and piroxicam (**18**), could generate both singlet oxygen and superoxide; however, generation of singlet oxygen seemed to be predominant, at least in the present chemical panel. In contrast, ROS generation was negligible or weak with most non-phototoxic chemicals. In accordance with the validated criteria for ROS data [25 (ΔA_{440 nm} · 10³) for singlet oxygen and 20 (ΔA_{560 nm} · 10³) for superoxide] (Onoue et al., 2008a), 16 testable photoallergens (ca. 70% of total photoallergens) could be correctly identified in the ROS assay, and positive predictions could also be made for 7 untestable photoallergens upon optional application of the mROS assay. The photoallergenic potential of musk xylene (**15**) could be predicted by the ROS/mROS assay based on the significant ROS generation [singlet oxygen (ΔA_{440 nm} · 10³), 104 ± 3; and superoxide (ΔA_{560 nm} · 10³),

Table 2
Photosafety characterization.

No.	Chemical name	Maximal MEC (M ⁻¹ cm ⁻¹) ^{a)}	ROS/mROS ^{b)}		3T3 NRU PT (PIF)	Photosafety information	
			Singlet oxygen (ΔA _{440 nm} · 103)	Superoxide (ΔA _{560 nm} · 10 ³)		Photoirritant	Photoallergenic
Photoallergens							
1	6-Methylcoumarin	7,502 [290 nm]	196±11	112±8	21.3	+	+
2	8-Methoxypsoralen	12,564 [299 nm]	143±13	8±8	120.5	+	+
3	Benzophenone	1,305 [290 nm]	227±21	<1	10.0	+	+
4	Bithionol	13,253 [307nm]	273±22	<1	3.6	+	+
5	Dichlorophene	5,373 [290 nm]	[213±4]	[<1]	1.9		+
6	Enoxacin	12,063 [345 nm]	516±30	271±24	119.0	+	+
7	Fenticlor	10,002 [303 nm]	[193±3]	[<1]	1.4		+
8	Glibenclamide	1,435 [298 nm]	37±12	<1	1.1		+
9	Hexachlorophene	8,381 [298 nm]	437±5	<1	1.0	–	+
10	Hydrochlorothiazide	4,121 [316 nm]	171±2	4±7	1.0		+
11	Indomethacin	8,738 [290 nm]	29±13	168±18	1.5	+	+
12	Isoniazid	1,251 [290 nm]	51±12	95±3	1.0		+
13	Ketoprofen	2,780 [290 nm]	316±8	106±5	75.4	–	+
14	Musk ambrette	2,755 [290nm]	[224±2]	[<1]	104.2	–	+
15	Musk xylene	448 [290 nm]	[104±3]	[38±7]	29.1	–	+
16	Octyl dimethyl PABA	28,879 [310 nm]	[76±3]	[158±9]	2.7	–	+
17	Omadine Na	6,519 [290 nm]	239±6	52±6	2.9		+
18	Piroxicam	22,389 [327 nm]	181±29	204±17	1.0		+
19	Pyridoxine HCl	7,539 [291 nm]	421±2	98±2	3.4	+	+
20	Sulfanilamide	3,677 [290 nm]	265±19	41±4	1.0		+
21	Sulfasalazine	33,116 [366 nm]	51±10	19±5	1.0		+
22	Tribromsalan	21,061 [325nm]	[81±2]	[<1]	49.0	+	+
23	Triclocarban	3,342 [290 nm]	[63±2]	[<1]	1.0	–	+
Non-phototoxic/non-photoallergenicchemicals							
24	4'-Methylbenzylidene camphor	11,876 [299 nm]	[<1]	[<1]	1.0	–	–
25	Aspirin	193 [290 nm]	3±2	<1	1.0		
26	Benzocaine	17,025 [290 nm]	4±1	9±1	1.0	–	–
27	Erythromycin	< 10	<1	<1	1.0	–	–
28	Methylsalicylate	4,722 [308 nm]	25±12	<1	1.0	–	–
29	Penicillin G	31 [318 nm]	20±24	9±21	1.0	–	–
30	Phenytoin	< 10	1±1	<1	1.0		

Black cells: over threshold in MEC and ROS/mROS or phototoxicity predicted in 3T3 NRU PT; and gray cells: probable phototoxicity in 3T3 NRU PT. a) Plain numbers: MEC values at λ_{max} observed between 290–700 nm; and italic numbers: MEC values at 290 nm (shoulders). b) Plain numbers: ROS data at 200 μM; plain numbers in brackets: mROS assay at 200 M; and italic letters in brackets: mROS assay at 100 μM. Data represent mean ± SD of three repeated experiments.

38 ± 7}, in spite of the weak UV/VIS-absorbing character of this compound. Although slight generation of singlet oxygen was seen in the case of methylsalicylate (**28**), other non-phototoxic chemicals were correctly judged to be negative. The ROS and optional mROS assays did not provide any false-negative predictions in the present investigation, and photoallergenic prediction of all test chemicals could be achieved with values of individual specificity, positive predictivity and negative predictivity of 85.7%, 95.8% and 100%, respectively (Table 3). These observations suggested that the ROS/mROS assays are useful for photoallergenicity screening, although careful consideration would be required to eliminate false-positive predictions.

3.3. 3T3 NRU PT

In addition to the photochemical screening systems (UV and ROS assays), the photoallergenic potential of all chemicals was also tested

using the 3T3 NRU PT (Table 2), which has been employed to predict photoirritation potential on the basis of PIF values of tested chemicals. PIF can be calculated from the cell viability curve and half-maximal inhibitory concentration (IC₅₀) values in the presence and absence of light, and the PIF values are indicative of photosafety ["no photoirritant" (PIF < 2), "probable photoirritant" (2 ≤ PIF < 5), and "photoirritant" (PIF ≥ 5)]. Although appropriate PIF criteria for prediction of photoallergenicity have not been established, these values were tentatively employed for the present study. Among the chemicals tested, 10 photoallergenic chemicals with no photoirritant potential (**5**, **7–10**, **12**, **18**, **20–21**, and **23**), as well as all non-phototoxic/non-photoallergenic chemicals, were identified as negatives because of their low PIF values (<2). In contrast, 12 photoallergens (**1–4**, **6**, **13–17**, **19**, and **22**) were captured by the 3T3 NRU PT, and, among them, 7 chemicals (**1–4**, **6**, **19**, and **22**) were recognized as photoirritants. Since the 3T3 NRU PT was designed for the identification

Table 3
Predictive capacity of ROS/mROS assays and 3T3 NRU PT.

	UV	ROS/mROS assays	3T3 NRU PT
Sensitivity (%)	95.7	100.0	52.2
Specificity (%)	57.1	85.7	100.0
Positive predictivity (%)	88.0	95.8	100.0
Negative predictivity (%)	80.0	100.0	38.9

of photoirritation, not for photoallergenicity (Onoue et al., 2009), the results of positive predictions for 7 photoirritant/photoallergenic chemicals (1–4, 6, 19, and 22) and negative predictions for 10 photoallergens (5, 7–10, 12, 18, 20–21, and 23) are not necessarily surprising. Nevertheless, the finding that 5 photoallergens (13–17) were positive even in the 3T3 NRU PT is interesting, suggesting that cytotoxicity via an unspecified mechanism might be involved in the pathway of photoallergenicity, or that the photoallergy-related biochemical responses could be partly detected by the 3T3 NRU PT. The sensitivity, individual specificity, positive predictivity and negative predictivity of the 3T3 NRU PT in the present study were calculated to be 52.2%, 100%, 100% and 38.9%, respectively (Table 3). These findings suggested rather limited predictive performance of the 3T3 NRU PT for photoallergenic potential.

4. Discussion

In the present study, three *in vitro* photosafety screening systems, UV/VIS spectral analysis, ROS assay, and 3T3 NRU PT, were applied to a panel of 30 selected chemicals to clarify their predictive performance for photoallergenic potential. These assay systems were found to be partly applicable for prediction of photoallergenicity, and their predictive capacity was ranked as follows: (i) sensitivity: ROS assay > UV/VIS absorption > 3T3 NRU PT, and (ii) specificity: 3T3 NRU PT > ROS assay > UV/VIS absorption. Thus, the 3T3 NRU PT seems unreliable for photoallergenicity prediction, at least with the current chemical set, whereas the *in vitro* photochemical assays could predict photoallergenicity with some false-positives.

In any type of phototoxic event, penetration and absorption of light in the skin, eyes, or other UV-exposed tissues can be a critical factor for triggering phototoxic cascades, and the absorption of photon energy by the phototoxin results in excitation of the molecule itself (Onoue et al., 2013b). Since molecular oxygen can act as the predominant acceptor of excitation energy, energy can be transferred from photo-excited chemicals to oxygen through type II photochemical reaction, resulting in the generation of singlet oxygen. Transfer of an electron or hydrogen could also lead to the formation of free radical species such as superoxide, peroxy radicals or reactive hydroxyl radical through a type I photochemical reaction. Thus, photo-excitation of chemicals tends to produce ROS, which may be one of major causative agents of phototoxic events (Brendler-Schwaab et al., 2004; Epstein and Wintroub, 1985). On the basis of the possible mechanisms and outcomes of phototoxic events, drug-induced phototoxic skin responses can be categorized into at least three types: (i) photoirritation through oxidative damage to cellular lipids and proteins, (ii) photoallergenicity through formation of photoantigens, and (iii) photogenotoxicity through DNA damage (Epstein and Wintroub, 1985; Onoue and Tsuda, 2006).

In these pathways, the photoexcitability of applied chemicals would be a key determinant for initiation of phototoxic responses; therefore, examination of photochemical properties might be a reasonable approach for prediction of photoallergenicity. In the present study, most photoallergens were successfully identified by both UV/VIS spectral analysis and ROS assay. These assays tended to overestimate the photoallergenic potential of test chemicals, as evidenced by some false-positive predictions: 3 chemicals in the UV/VIS spectral analysis, and 1 chemical in ROS assay. Not all photo-excited chemicals will induce photochemical toxicity, because some chemicals in the excited state

could release the absorbed photon energy via emission of fluorescence, phosphorescence or heat, and thereby return to their ground state (Seto et al., 2012). This might partly explain the limited specificity of the UV/VIS approach. In theory, the ROS assay can also provide false-positive predictions, since it may capture all photochemically active substances (Onoue and Tsuda, 2006). Some photolabile substances would be judged as positive in the ROS assay if they are potent ROS generators in their photodegradation pathways. In addition to the false-positives, a false-negative prediction was made for one photoallergen in the UV/VIS approach, although this chemical exhibited potent ROS generation in the ROS assay and photodynamic cytotoxicity in the 3T3 NRU PT. A previous study also demonstrated that some phototoxic cosmetic ingredients have subthreshold MEC values ($<1000 \text{ M}^{-1} \text{ cm}^{-1}$), providing false-negative predictions in UV/VIS spectral analysis (Onoue et al., 2013c). The MEC criterion value was well validated, at least for photosafety screening of pharmaceutical substances (Bauer et al., 2014; Henry et al., 2009); however, a reduced threshold value might be needed to enable reliable photosafety testing of non-pharmaceutical chemicals. Further validation studies using a wide variety of model chemicals will be needed to ensure the applicability of these photochemical assay systems for photoallergenicity assessment.

3T3 NRU PT was originally developed to detect photoirritant potential, and is not designed to predict other phototoxic events, such as photoallergenicity and photogenotoxicity (Onoue et al., 2009); therefore, the limited predictive performance of 3T3 NRU PT in photoallergenicity testing is not surprising. Also, 3T3 NRU PT may make false-negative predictions for chemicals predominantly absorbing in the UVB range, since only a UVA light source is used in the assay to avoid the cytotoxic effect of UVB light on 3T3 cells (Ceridono et al., 2012). According to the Clog *P* values of tested chemicals (Table 1), some photoallergens would be poorly soluble in aqueous medium, possibly providing negative predictions in the 3T3 NRU PT. Thus, in comparison with the photochemical assays, the 3T3 NRU PT might be less effective for evaluation of photoallergenicity. The exact reason why 3T3 NRU PT can make positive predictions for at least some photoallergenic chemicals remains unclear, and further mechanistic investigations might provide a basis for modification of the assay to improve its applicability for photoallergenicity screening. It is also important to accelerate the development of new *in vitro* photosensitization assays, which should be based on the photosensitization AOP (Adverse Outcome Pathway).

In conclusion, our findings indicate that photochemical assays, in particular the ROS assay, are applicable for the prediction of photoallergenic potential. Although there were some false-positive predictions, the photochemical assays show high sensitivity for assessment of photoallergenicity. However, a better understanding of their limitations through careful validation studies would be of great value for avoiding overestimation and misleading conclusions.

Conflict of interest

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

Transparency document

The Transparency document associated in with this article can be found, in online version.

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