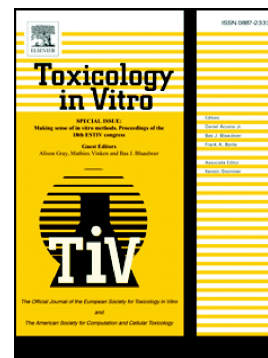


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***In chemico* sequential testing strategy for assessing the photoallergic potential**

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Abstract

Several non-animal testing methods to assess photoallergic potential have been developed so far, while none of them have yet to be validated and regulatory accepted. Currently, some photoreactivity assays such as UV-VIS spectral analysis and ROS assay are generally used for initial photosafety assessments because of their high sensitivity. However, they have a low specificity, generating a high percentage of false positive results, and the development of a follow-up assessment method is desired. Therefore, this study aimed to develop an *in chemico* photoallergy testing method, photo-direct peptide reactivity assay (DPRA). Based on photosafety information, 34 photoallergens and 15 non-photoallergens were selected and subjected to UV-VIS spectral analysis, ROS/micellar ROS assays, photo-DPRA, sequential testing strategy (STS) consisting of all three methods, and 3T3 neutral red uptake phototoxicity testing (3T3 NRU PT). Combination of the methods addressing the key events of photoallergy exhibited high prediction performance. Our results showed the proposed strategy would be useful to predict the photoallergic potential of chemicals as the follow-up assessment for false positive chemicals by UV/VIS spectral analysis and ROS assay.

Key words:

Photoallergy, Phototoxicity, UV-VIS spectral analysis, ROS assay, Photo-DPRA, STS

Abbreviations:

UV: ultraviolet, VIS: visible light, ROS: Reactive oxygen species, DPRA: Direct peptide reactivity assay, IATA: Integrated approaches to testing and assessment, STS: Sequential testing strategy, OECD: the Organisation for Economic Cooperation and Development, KE: Key event, AOP: adverse outcome pathway, ICH: the International Council on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, MEC: Molar extinction coefficient

1. Introduction

Photoallergy is an immune mediated reaction that occurs when a photoallergen is applied orally or dermally and subsequently exposed to ultraviolet (UV) radiation, and it can sometimes be caused by cosmetic and pharmaceutical ingredients. To predict the photoallergic potential of chemicals, guinea pig methods such as the Adjuvant and Strip method (Sato et al., 1980) and Herber method (Herber, 1969), have been generally used. In the last decade, however, the safety assessment of chemicals based exclusively on non-animal testing methods are needed from the viewpoint of regulatory requirements and ethical issues.

The main sequential events in photoallergic dermatitis are virtually the same as those of ordinary allergic contact dermatitis, except for the requirement of UV irradiation both in the sensitization and elicitation phases (Tachura, 2018). The adverse outcome pathway (AOP) for skin sensitization was recently proposed, and the development of non-animal testing methods covering three key events (KEs: KE1: binding of haptens to endogenous skin proteins, KE2: keratinocyte activation and KE3: dendritic cell activation) of the AOP (OECD, 2012) has been accelerated. These three KEs are addressed by the following methods, which have been validated and adopted by the Organisation for Economic Cooperation and Development (OECD) for regulatory purposes: the direct peptide reactivity assay (DPRA) addressing KE1 (OECD, 2019a); the ARE-Nrf2 luciferase test method (KeratiNoSens™ assay) representing KE2 (OECD, 2018a); and the human Cell Line Activation Test (h-CLAT) describing KE3 (OECD, 2019a). On the other hand, although several methods such as photo-DPRA (Patel et

al., 2019), photo-KeratinoSensTM (Tsujita-Inoue et al., 2015) and photo-h-CLAT (Hino et al., 2008; Hoya et al., 2009) have been reported, there is still no validated and regulatory accepted non-animal alternative methods to assess photoallergic potential of chemicals. Furthermore, there is no scientific consensus that a single alternative testing method can serve as a stand-alone assay to fully replace animal testing because of the complexity of the process involved in the generation of photoallergy. Therefore, integrated approaches to testing and assessment (IATA) using the combination of several assays representing different KEs of the AOP is required as is the case with skin sensitization assessment.

According to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) S10 guideline (ICH, 2014), photoreactivity assays such as UV-VIS spectral analysis (OECD, 1981) and ROS assay (OECD, 2019b) are recommended to be used as screening method for the initial photosafety evaluation. Although both assays have high sensitivity for predicting direct *in vivo* phototoxic and/or photoallergic chemicals, they have a low specificity, generating a high percentage of false positive results. A negative result would indicate a very low risk, whereas a positive result would be a flag for follow-up assessment. Currently, there is still no standardized *in chemico/in vitro* photoallergy method, and hence this study aimed to develop the *in chemico* photoallergy testing method, photo-DPRA. The predictive capacity of an *in chemico* sequential testing strategy consisting of UV-VIS spectral analysis, ROS/mROS assay and photo-DPRA for evaluating photoallergic potential were also assessed using chemicals with a

wide structural diversity.

2. Materials and methods

2.1. Test chemicals

Based on published photosafety information, 50 chemicals (34 known photoallergens and 16 non-photoallergens) were used in this study, as listed in Table 1.

2.2. Irradiation conditions

The photo-DPRA and ROS/mROS assay were conducted using an Atlas Suntest CPS Plus (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 was installed to adapt the spectrum of the artificial light source to that of natural day-light, and the Atlas Suntest CPS series had a high irradiance capability that met CIE85/1989 daylight simulation requirements. The irradiation was carried out at 25°C with an irradiance of ca. 2.0 mW/cm² as determined with a calibrated UVA detector Dr. Hönle#0037 (Dr. Hönle, München, Germany).

2.3. Photo-DPRA

2.3.1. Assay procedure

The photo-DPRA was performed based on OECD Test Guideline 442C (OECD, 2019a) with the addition of UV-VIS light irradiation. Peptides containing cysteine (Ac-RFAACAA-COOH) or lysine (Ac-RFAAKAA-COOH) were obtained from RS synthesis (Louisville, KY, USA). Briefly, a 0.67 mM peptide stock solution was prepared in 100 mM sodium phosphate buffer (pH7.5) for the cysteine peptide or 100 mM ammonium acetate

buffer (pH10.2) for the lysine

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Table 1 Chemicals used in this study

Chemical name	CAS No.	MW (g/mol)	Supplier	Photosafety information		Reference
				Photoallergy	Photoirritantion	
Photoallergens						
1 3,3',4',5-Tetrachlorosalicylanilide	1154-59-2	351.0	Kanto chemical	+	+	Onoue at al., 2017
2 3,5,4'-Tribromosalicylanilide	87-10-5	449.9	Fujifilm-Wako	+	+	Onoue at al., 2017
3 4-Methyl-7-ethoxycoumarin	87-05-8	204.2	Fujifilm-Wako	-	ND	Onoue at al., 2017
4 6-Methylcoumarin	92-48-8	160.2	Santa Cruz Biotechnology	+	+	Onoue at al., 2017
5 7-Methoxycoumarin	531-59-9	176.2	Fujifilm-Wako	+	ND	Onoue at al., 2017
6 8-Methoxypsoralen	298-81-7	216.2	Nacalai tesque	+	+	Onoue at al., 2017
7 Benzophenone	119-61-9	182.2	Sigma-Aldrich	+	+	Onoue at al., 2017
8 Bithionol	97-18-7	356.0	Tokyo Chemical Industry	+	+	Onoue at al., 2017
9 Chlorpromazine HCl	69-09-0	355.3	LKT laboratories	+	+	Onoue at al., 2017
10 Dichlorophene	97-23-4	267.1	Sigma-Aldrich	+	ND	Onoue at al., 2017
11 Diclofenac Na	15307-79-6	318.1	Fujifilm-Wako	+	+	Onoue at al., 2017
12 Enoxacin	74011-58-8	320.3	Toronto research chemicals	+	+	Onoue at al., 2017
13 Fenticlor	97-24-5	287.2	Tokyo Chemical Industry	+	+	Onoue at al., 2017
14 Furosemide	54-31-9	330.7	Sigma-Aldrich	+	+	Onoue at al., 2017
15 Griseofulvin	126-07-8	352.8	Tokyo chemical industry	+	+	Onoue at al., 2017
16 Hexachlorophene	70-30-4	406.9	Nacalai tesque	+	-	Onoue at al., 2017
17 Hydrochlorothiazide	58-93-5	297.7	Tokyo Chemical Industry	+	+	Onoue at al., 2017
18 Isoniazid	54-85-3	137.1	LKT laboratories	+	ND	Onoue at al., 2017
19 Ketoprofen	22071-15-4	254.3	Fujifilm-Wako	+	+	Onoue at al., 2017
20 Lomefloxacin HCl	98079-52-8	387.8	Tokyo Chemical Industry	+	+	Onoue at al., 2017

21	Mequitazine	29216-28-2	322.5	Fujifilm-Wako	+	ND	Onoue et al., 2017
22	Musk ambrette	83-66-9	268.3	Tokyo Chemical Industry	+	-	Onoue et al., 2017
23	Musk xylene	81-15-2	297.3	Tokyo Chemical Industry	+	-	Onoue et al., 2017
24	Octyl dimethyl PABA	21245-02-3	277.4	Sigma-Aldrich	+	-	Onoue et al., 2017
25	Ofloxacin	82419-36-1	361.4	Sigma-Aldrich	+	+	Onoue et al., 2017
26	Omadine Na	3811-73-2	149.1	Alfa Aesar	+	ND	Onoue et al., 2017
27	Piroxicam	36322-90-4	331.3	Sigma-Aldrich	+	+	Onoue et al., 2017
28	<i>p</i> -Phenylenediamine	106-50-3	108.1	Tokyo Chemical Industry	-	ND	Onoue et al., 2017
29	Promethazine HCl	58-33-3	320.9	Fujifilm-Wako	+	+	Onoue et al., 2017
30	Pyridoxine HCl	58-56-0	205.6	Sigma-Aldrich	+	+	Onoue et al., 2017
31	Quinine	130-95-0	324.4	Fujifilm-Wako	+	+	Onoue et al., 2017
32	Sulfanilamide	63-74-1	172.2	Fujifilm-Wako	+	ND	Onoue et al., 2017
33	Sulfasalazine (Salazosulfapyridine)	599-79-1	398.4	Fluka	+	ND	Onoue et al., 2017
34	Tetracycline HCl	64-75-5	480.9	Fujifilm-Wako	+	+	Onoue et al., 2017
Non-photoallergens							
35	1,3-Butylene glycol	107-88-0	90.1	Nacalai tesque	-	-	Onoue et al., 2013b
36	4-Methyl benzylidene camphor	36861-47-9	254.4	Alfa Aesar	-	-	Onoue et al., 2013b
37	Ascorbic acid	50-81-7	176.1	Sigma-Aldrich	-	-	Onoue et al., 2013b
38	Aspirin	50-78-2	180.2	Fujifilm-Wako	-	-	Onoue et al., 2013a
39	Benzocaine	94-09-7	165.2	Fujifilm-Wako	-	-	Onoue et al., 2013a
40	Cinnamic acid	140-10-3	148.2	Fujifilm-Wako	-	-	Bickers et al., 2005
41	Cinnamic aldehyde	104-55-2	132.2	Sigma-Aldrich	-	-	Bickers et al., 2005
42	Erythromycin	114-07-8	733.9	Fujifilm-Wako	-	-	Onoue et al., 2013a
43	Glycerin	56-81-5	92.1	Sigma-Aldrich	-	-	Onoue et al., 2013b
44	Lactic acid	50-21-5	90.1	Sigma-Aldrich	-	-	Onoue et al., 2013b

45	L-histidine	71-00-1	155.2	Fujifilm-Wako	-	-	Onoue et al., 2013a
46	Methyl salicylate	119-36-8	152.2	Fujifilm-Wako	-	-	Onoue et al., 2013b
47	Octyl salicylate	118-60-5	250.3	Tokyo Chemical Industry	-	-	Seto et al., 2013
48	Penicillin G	61-33-6	334.4	Sigma-Aldrich	-	-	Onoue et al., 2013b
49	Phenytoin	57-41-0	252.3	Fujifilm-Wako	-	-	Onoue et al., 2016
50	Sulisobenzone	4065-45-6	308.3	Sigma-Aldrich	-	-	Onoue et al., 2013b

MW, molecular weight; ND, not determined.

peptide, and a 20 mM (for cysteine assay) or 100 mM (for lysine assay) test chemical stock solution was prepared with acetonitrile, water, isopropanol, acetone or 1:1 mixture of acetonitrile and buffer. In addition, the buffer solution and vehicles used for cysteine assay were purged with argon gas in order to avoid auto-oxidation of cysteine. The final reaction mixture, containing 0.5 mM of the peptide and 5 mM (for cysteine assay) or 25 mM (for lysine assay) of the test chemical, was prepared in glass vials. All glass vials were sealed with microplate seal (UV pass-through type) and irradiated using the Atlas Suntest CPS plus (irradiation condition) or kept in the dark (non-irradiation condition). After irradiation, both peptides were quantified using an Alliance e2695 HPLC (Waters, Milford, MA, USA) equipped with a Waters 2998 Photodiode Array Detector and a Zorbax SB-C18 (100 mm × 2.1 mm; particle size 3.5 µm; Agilent Technologies, Waldbornn, Germany). Peptides were detected at 220 nm. The percentage of depletion was determined based on the decrease in the non-reacted peptide concentration in the sample relative to the average concentration measured in the control.

2.3.2. Dose finding assay

The final reaction mixtures were prepared in the same way as described in 2.3.1. Assay procedure and incubated in the dark for 24 hours at 25°C. When the cysteine or lysine % depletion was greater than 13.89%, the dose finding assay was reconducted to determine the highest concentration showing less than 13.89% depletion for each chemical. The cut-off criteria were set based on TG442C because the main sequential events in photoallergy are

virtually the same as those of ordinary skin sensitization except for the requirement of UV irradiation. In cases where co-elution occurs only with the cysteine peptide, the criterion of lysine is not described in TG442C and thus 13.89% was employed in the same way as cysteine prediction model.

2.3.3. Main study

The final reaction mixtures were prepared in triplicate in the same way as described in 2.3.1. Assay procedure. All glass vials were sealed with microplate seal (UV pass-through type) and exposed to a dose of 5 J/cm² (irradiation condition) or kept in the dark (non-irradiation condition). After irradiation, the peptide was quantified to determine the percentage of depletion and calculate the difference in % depletion values with UV (UV+) and without UV (UV-) exposure (hereinafter refer to Δ Cys or Δ Lys). When the mean of Δ Cys and Δ Lys % depletion was greater than 6.38%, the test chemical was predicted as positive. If the test chemical was co-eluted with lysine peptide, 13.89% was used as the positive criteria instead (Table 2A and 2B).

Table 2 Photo-DPRA (A) Cys1:10 / Lys1:50 prediction mode (B) Cys1:10 prediction model

(In case co-elution occurred)

(A) Mean of Δ Cys and Δ Lys (%) Prediction	
≤ 6.38	Negative
> 6.38	Positive
(B) Δ Cys (%) Prediction	

≤ 13.89	Negative
> 13.89	Positive

2.4. UV-VIS spectral analysis

The test chemical was dissolved in methanol, ethanol, water or acetonitrile, and UV-VIS absorption spectra (290–700 nm) was measured using a UV-VIS Multipurpose Spectrophotometer MPS-2400 (Shimadzu, Kyoto, Japan) interfaced to a PC for data processing (software; UV Probe Version 1.12). The sample solution was transferred to a spectrofluorimeter quartz cell with 10 mm pathlength and then the UV-VIS spectra was recorded. Based on the spectrum data, the molar extinction coefficient (MEC) value was calculated at its $\lambda_{\max}$ (290-700 nm).

2.5. ROS assay/micellar ROS (mROS) assay

The ROS assay was conducted as described in the OECD Test Guideline 495 (OECD, 2019b) with minor modifications. Briefly, the test compound was dissolved in dimethyl sulfoxide at 10 mM as a stock solution. Each test compound (200 μM), *p*-nitrosodimethylaniline (50 μM) and imidazole (50 μM) were dissolved in 20 mM NaPB (pH7.4) to determine singlet oxygen generation. Each test compound (200 μM) and nitroblue tetrazolium (50 μM) were dissolved in 20 mM NaPB (pH7.4) to measure superoxide generation. When the test chemical could not be dissolved due to limited solubility, a micellar solution of 0.5% Tween 20 was added to the assay mixtures and experimental controls (micellar ROS assay). A plastic 96-well microplate (clear, untreated, flat-bottomed; Asahi

Glass, Tokyo, Japan) containing reaction mixtures was fixed in the reaction container with a quartz cover (Ozawa Science, Aichi, Japan) and irradiated for 1 hour. Before and after exposure to artificial sunlight, absorbances at 440 and 560 nm were monitored using a SYNERGY H1 microplate reader (BioTek, Winooski, VT, USA). To avoid false predictions due to spectral interference at 440 nm for singlet oxygen and at 560 nm for superoxide, experimental controls in which the test substance (200 μ M) in 20 mM NaPB (pH7.4) were added, and the control values were subtracted from the sample values. The photoreactivity of each tested substance should be judged as follows, based on the mean value of triplicate: (i) positive for singlet oxygen ($\Delta A_{440\text{nm}} \times 10^3$): 25 or more; and/or superoxide ($\Delta A_{560\text{nm}} \times 10^3$): 20 or more, or (ii) negative for singlet oxygen ($\Delta A_{440\text{nm}} \times 10^3$): less than 25, and superoxide ($\Delta A_{560\text{nm}} \times 10^3$): less than 20.

2.6. *In vitro* 3T3 NRU PT

Publicly available data was collected from (Schmidt et al., 2019). The 3T3 neutral red uptake phototoxicity test (3T3 NRU PT) is a validated method for evaluating the phototoxic potential of chemical and adopted in the OECD Test Guideline 432 (OECD, 2019c). This method is considered as an initial test for phototoxicity in the ICH S10 guideline.

3. Results

3.1. Confirmation of peptide stability

The effect of irradiation on cysteine and lysine peptides was studied. The 0.5 mM peptide solutions were prepared and irradiated at a dose of 0, 5, 10 or 20 J/cm². The results showed

both peptides are stable until irradiated at a dose of 20 J/cm² (data not shown). The effect of vehicles such as acetonitrile, water, isopropanol and acetone that are typically used for original DPRA was also confirmed at a dose of 5 J/cm². Only when acetone was used for the cysteine assay, the irradiation did cause cysteine peptide depletion (Fig. 1). Therefore, acetone was used only for the lysine assay.

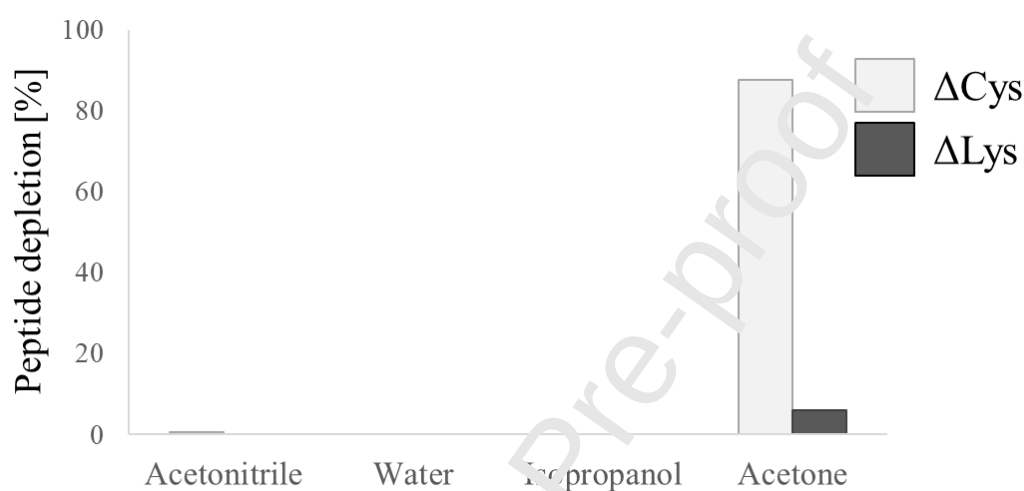


Fig.1. The effect of solvent vehicles on cysteine and lysine peptide.

3.2. Selection of the irradiation dose

A suitable irradiation dose (5 J/cm²) was selected based on the results shown in Fig. 2. Reaction mixtures containing 0.5 mM of the peptides and 5 mM of the test chemicals (6-methylcoumarin, hydrochlorothiazide, isoniazid, musk ambrette, musk xylene or sulfanilamide used for the positive controls; 4-methyl benzylidene camphor, erythromycin, methyl salicylate, penicillin G, phenytoin or octyl salicylate used for the negative controls) were prepared and irradiated at a dose of 0, 5 or 10 J/cm². At a dose of 5 J/cm², all positive control chemicals except isoniazid were above the criterion of the Cys1:10 prediction model (13.89%), while all negative control chemicals were below its criterion. At a dose of 10 J/cm²,

4 out of 6 negative controls exceeded its criterion, and thus 5 J/cm² was selected as a suitable irradiation dose.

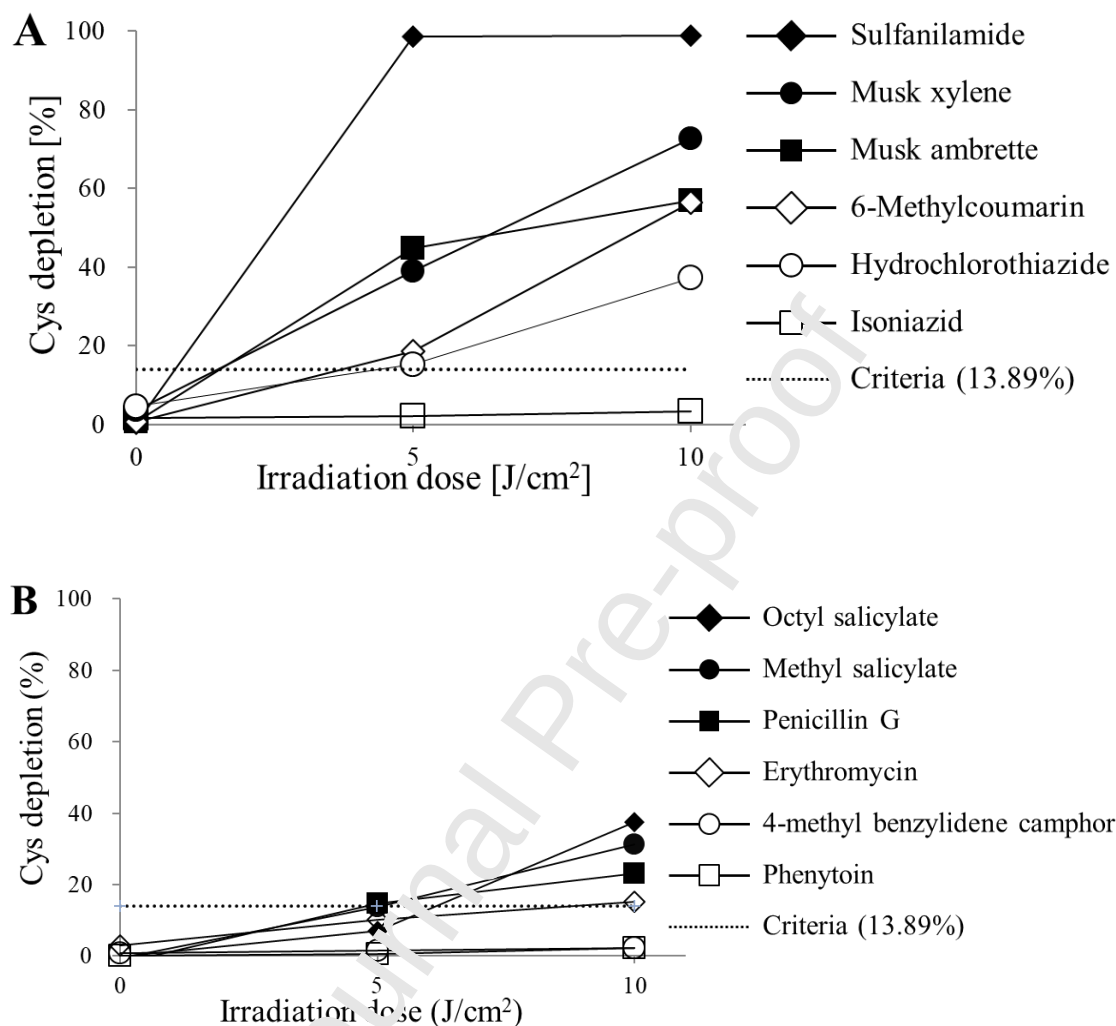


Fig.2 The relationship between Cys depletion and irradiation dose when using (A) photoallergens or (B) non-photoallergens.

3.3. Confirmation of the dose-response reaction without irradiation

Chemicals with peptide reactivity like skin sensitizers typically react with cysteine and/or lysine peptides without irradiation, which can cause the underestimation of photoreactivity assessment. Therefore, the dose-response reaction of cinnamaldehyde (skin sensitizer and

non-photoallergen) and fenticlor (photoallergen) which react with peptides even in a non-irradiated condition was observed (Fig. 3). The reaction mixtures, containing 0.5 mM of cysteine peptides and the test chemical at 6 concentrations of a 2-fold serial dilution, were prepared and irradiated at a dose of 5 J/cm². The same dose-response curves were observed with and without irradiation for cinnamaldehyde, while entirely different dose-response curves were obtained for fenticlor. In addition, there were no significant differences with Δ Cys depletion when using 5 mM of fenticlor. Therefore, when cysteine or lysine % depletion was greater than 13.89% without irradiation, the dose finding assay was reconducted to determine the highest concentration showing less than 13.89% depletion for each chemical.

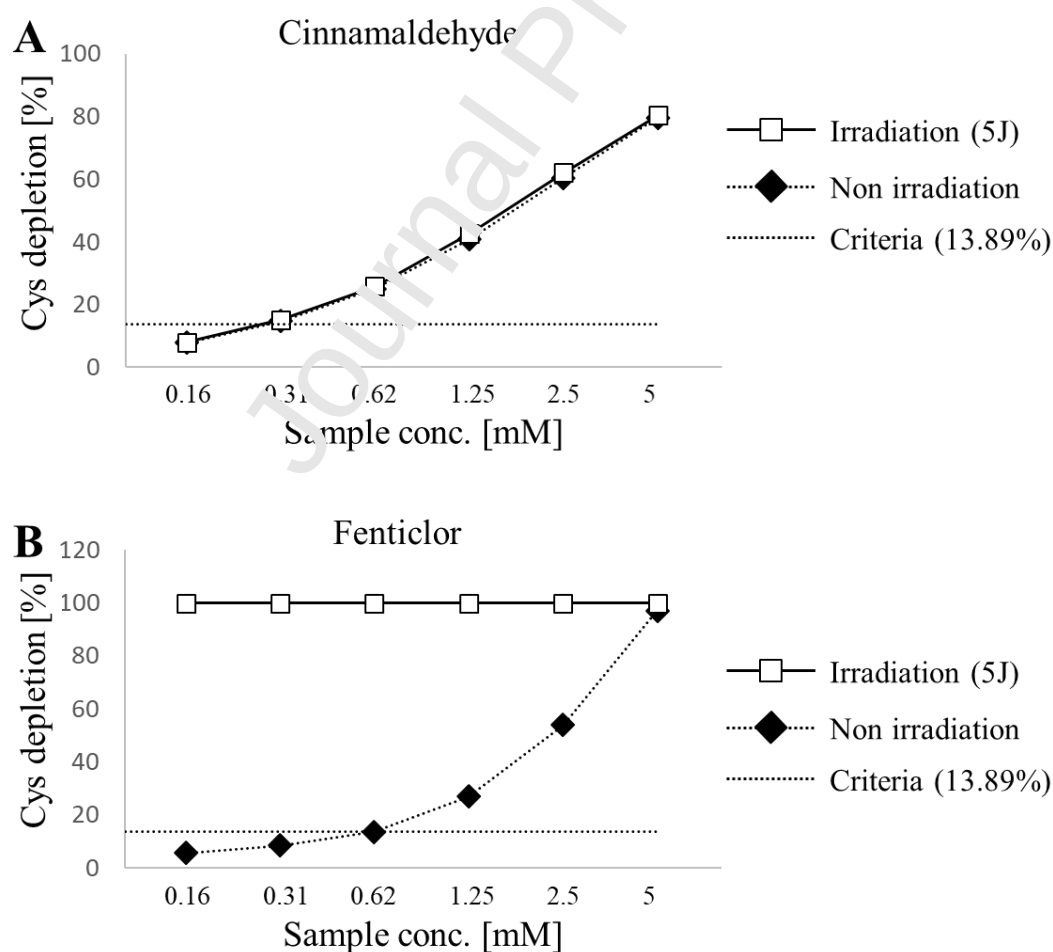


Fig. 3 Dose-response reaction with and without irradiation using (A) cinnamaldehyde or (B) Fenticlor

3.4. Overall prediction results of photo-DPRA

The results of photo-DPRA are summarized in Table 3 and Table S1. Five out of 50 chemicals were not applicable due to co-elution with cysteine peptide. Based on the criteria in TG442C (Table 2A and 2B), the sensitivity, specificity and accuracy were calculated as 93.8%, 84.6% and 91.1%, respectively. All positive chemicals showed more than 13.89% of Δ Cys depletion, while there was no chemical showing more than 13.89% of Δ Lys depletion. Further studies should be conducted to clarify the applicability domain and accumulate a reference dataset for developing complete AOP-based TAA approaches.

Table 3 Predictive performance of photo-DPRA

	Photo-DPRA		
	Positive	Negative	Total
Photoallergen	30	2	34
Non-photoallergen	2	11	16
Total	32	13	50
		Sensitivity	93.8% (30/32)
		Specificity	84.6% (11/13)
		Accuracy	91.1% (41/45)

3.5. Sequential testing strategy (STS) for assessing photoallergic potential

The proposed sequential testing strategy (STS) is illustrated in Fig. 4. The initial consideration for the assessment of photoallergic potential is photochemical property. If the chemical does not have a MEC greater than $1000 \text{ L mol}^{-1} \text{ cm}^{-1}$, no further testing is needed.

For compounds with MEC values of $1000 \text{ L mol}^{-1} \text{ cm}^{-1}$ or higher, negative ROS assay results could support a decision that no further assessment is warranted. For poorly water-soluble materials, mROS assay was optionally applied.

The overall results from the UV-VIS spectral analysis, ROS/mROS assay, photo-DPRA, STS and 3T3 NRU PT are summarized in Table 4 and Table 5. Both the UV-VIS spectral analysis and ROS/mROS assay showed high sensitivity, suggesting that these assays are useful as a screening tool. By contrast, the sensitivity of 3T3 NRU PT was 72.7% and it did not allow for a reliable prediction of photoallergic potential compared with photo-DPRA. The specificities of the UV-VIS spectral analysis and ROS/mROS assay are 56.3% or 75.0%, respectively, meaning that further tests would be required to avoid false positive predictions. Using the STS approach, 93.8% of the chemicals were correctly identified. Note that two chemicals (isoniazid and sulfasalazine) could not be detected and further assessment using other methods such as photo-KeratinsensTM and photo-h-CLAT might be needed.

Table 4 Summary of results from UV-VIS spectral analysis, ROS/mROS assay, photo-DPRA, STS and 3T3 NRU PT.

		Maximal MEC (L mol ⁻¹ cm ⁻¹)		ROS/	Photo-DPRA			STS	3T3 NRU
Chemical name		290-700nm	320-700nm	mROS	ΔCys	ΔLys	Result		PT ^e
Photoallergens									
1	3,3',4',5-Tetrachlorosalicylanilide	11204(290nm)	8063(323nm)	P ^a	98.8	10.4	P	P	P
2	3,5,4'-Tribromosalicylanilide	11669(290nm)	9433(327nm)	P ^a	97.3	5.2	P	P	P
3	4-Methyl-7-ethoxycoumarin	15588(321nm)	15588(321nm)	P ^a	72.6	1.5	P	P	P
4	6-Methylcoumarin	7540(290nm)	5436(320nm)	P ^a	16.6	0.1	P	P	P
5	7-Methoxycoumarin	15332(322nm)	15332(322nm)	P ^a	91.9	7.4	P	P	P
6	8-Methoxypsoralen	12566(299nm)	6472(320nm)	P ^a	74.4	4.2	P	P	P
7	Benzophenone	3748(290nm)	295(320nm)	P ^a	96.9	12.0	P	P	P
8	Bithionol	9319(307nm)	3185(320nm)	P ^a	91.5	12.3	P	P	P
9	Chlorpromazine HCl	3583(306nm)	2873(320nm)	P ^a	99.4	2.2	P	P	P
10	Dichlorophene	5401(290nm)	8020(320nm)	P ^a	99.3	7.1	P	P	N
11	Diclofenac Na	8746(290nm)	207(320nm)	P ^a	99.6	0	P	P	N
12	Enoxacin	11972(344nm)	11972(344nm)	P ^a	96	NA	P	P	P
13	Fenticlor	10039(300nm)	1949(320nm)	P ^a	98.4	9.0	P	P	N
14	Furosemide	6207(340nm)	6207(340nm)	P ^b	23.4	0.2	P	P	P
15	Griseofulvin	25136(290nm)	5555(320nm)	P ^c	75.8	1.0	P	P	N
16	Hexachlorophene	6184(298nm)	27(320nm)	P ^a	72.3	0.6	P	P	Equivocal
17	Hydrochlorothiazide	2446(317nm)	2375(320nm)	P ^c	12.1	1.4	P	P	N
18	Isoniazid	1216(290nm)	222(320nm)	P ^d	0	0.7	N	N	N
19	Ketoprofen	2779(290nm)	141(320nm)	P ^a	96.5	3.9	P	P	P
20	Lomefloxacin HCl	36755(290nm)	13685(320nm)	P ^c	NA	0	NA	NA	P

21	Mequitazine	3944(306nm)	2979(320nm)	P ^c	98.5	0	P	P	ND
22	Musk ambrette	2734(290nm)	1058(320nm)	P ^a	46.4	1.3	P	P	P
23	Musk xylene	450(290nm)	369(320nm)	P ^a	32.5	0.1	P	N	P
24	Octyl dimethyl PABA	29025(309nm)	20626(320nm)	P ^a	38.7	0.7	P	P	Equivocal
25	Ofloxacin	23204(390nm)	10173(320nm)	P ^b	90.3	0	P	P	P
26	Omadine Na	12860(290nm)	3640(320nm)	P ^d	95.3	1.2	P	P	Equivocal
27	Piroxicam	20820(326nm)	20820(326nm)	P ^a	17.0	0	P	P	N
28	<i>p</i> -Phenylenediamine	1884(308nm)	1428(320nm)	P	29.0	3.7	P	P	P
29	Promethazine HCl	4397(298nm)	2066(320nm)	P ^a	95.3	0	P	P	P
30	Pyridoxine HCl	7539(290nm)	1198(320nm)	P ^d	86.7	5.5	P	P	Equivocal
31	Quinine	5036(332nm)	5036(332nm)	P ^a	96.1	NA	P	P	P
32	Sulfanilamide	3634(290nm)	38(320nm)	P ^a	96.6	3.8	P	P	N
33	Sulfasalazine	33326(366nm)	33326(366nm)	P ^d	1.7	0	N	N	N
34	Tetracycline HCl	12973(356nm)	12973(356nm)	P ^a	NA	NA	NA	NA	P
Non-photoallergens									
35	1,3-Butylene glycol	0(290nm)	0(320nm)	N ^a	0	0	N	N	N
36	4-Methyl benzylidene camphor	25141(299nm)	12478(320nm)	N ^a	1.5	0.1	N	N	N
37	Ascorbic acid	5(290nm)	0(320nm)	P ^a	0.9	0	N	N	N
38	Aspirin	310(290nm)	5(320nm)	N ^b	NA	3.1	NA	N	N
39	Benzocaine	19465(292nm)	1577(320nm)	N ^b	NA	1.0	NA	N	N
40	Cinnamic acid	13843(290nm)	33(320nm)	P ^b	0.7	NA	N	N	N
41	Cinnamic aldehyde	4517(290nm)	277(320nm)	N	0.5	0.6	N	N	ND
42	Erythromycin	26(290nm)	3(320nm)	N ^b	4.7	0.7	N	N	N
43	Glycerin	0(290nm)	0(320nm)	N ^a	0	0	N	N	N
44	Lactic acid	0(290nm)	0(320nm)	N ^a	0	0	N	N	N

45	L-histidine	0(290nm)	0(320nm)	P ^b	0	0	N	N	N
46	Methyl salicylate	3973(306nm)	2095(320nm)	P ^a	14.4	3.2	P	P	N
47	Octyl salicylate	4331(306nm)	2392(320nm)	N ^c	11.1	0.7	N	N	N
48	Penicillin G	9(322nm)	9(322nm)	N ^a	15.7	NA	P	N	N
49	Phenytoin	0(290nm)	0(320nm)	N ^d	0.7	0	N	N	N
50	Sulisobenzone	10679(290nm)	7149(324nm)	N ^a	NA	0	NA	N	N

MEC, molecular extinction coefficient; P, positive; N, negative; NA, not applicable; ND, no data.

Black cells: mROS data (micellar solution of 0.5% Tween 20 was added to the assay mixture due to solubility issues)

^a Onoue et al.,2013b

^b Onoue et al.,2013a

^c Seto et al., 2013

^d Onoue et al., 2016

^e Schmidt et al., 2019

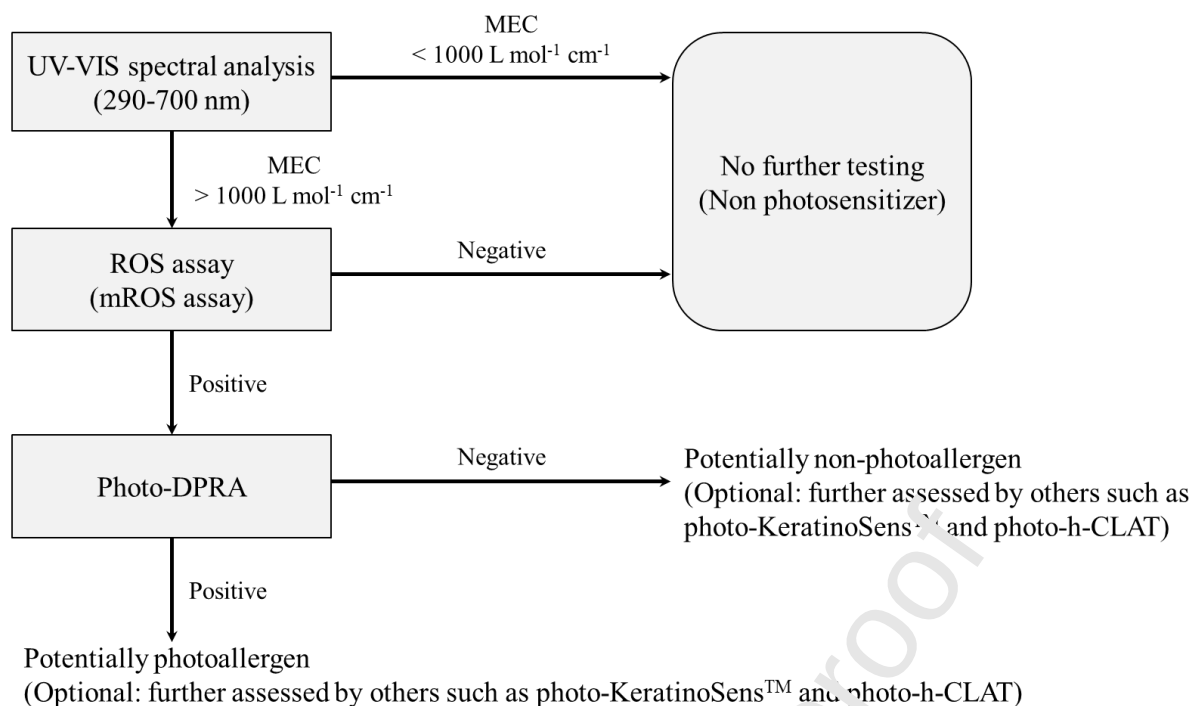


Fig. 4 Proposed *in chemico* sequential testing strategy consisting of UV-VIS spectral analysis, ROS/mROS assay and photo-DPRA for assessing photoallergic potential.

Table 5 Predictive capacity of UV/VIS spectral analysis, ROS/mROS assays, STS and 3T3 NRU PT.

	Sensitivity (%)	Specificity (%)	Accuracy (%)	N
UV-VIS	97.1 (33/34)	56.3 (9/16)	84.0 (42/50)	50
ROS/mROS assay	100 (33/34)	75.0 (12/16)	92.0 (46/50)	50
Photo-DPRA	93.8 (30/32)	84.6 (11/13)	91.1 (41/45)	45
STS	90.6 (29/32)	93.8 (15/16)	91.7 (44/48)	48
3T3 NRU	72.7 (24/33)	100 (15/15)	81.3 (39/48)	48

4. Discussions

In the ICH S10 guideline (ICH, 2014), four different effects (phototoxicity, photoallergy, photogenotoxicity and photocarcinogenicity) have been discussed in connection with photosafety testing, and the terminology phototoxicity and photoallergy is defined as follows:

- Phototoxicity (photoirritation): An acute light-induced tissue response to a photoreactive chemical.
- Photoallergy: An immunologically mediated reaction to a chemical, initiated by the formation of photoproducts (e.g., protein adducts) following a photochemical reaction.

Photosensitization is a general term occasionally used to describe all light-induced tissue reactions. Thus, in order to clearly distinguish between photoallergy and phototoxicity, the term photosensitization is not used in this text. Photochemical properties and photoreactivity are described as key characteristics of phototoxic and/or photoallergic chemicals (ICH, 2014) and the UV-VIS spectral analysis and ROS assay are recommended for evaluating the photoreactivity of drug candidates in the initial photosafety assessment. No further tests are needed if a negative result is obtained, whereas a positive result should not be regarded as indicative of a likely clinical photosafety risk, but rather as a flag for follow-up assessment.

According to the ICH S10 guideline, chemicals with an MEC of less than $1000 \text{ L mol}^{-1} \text{ cm}^{-1}$ are judged to be less photosafety risk, and all photoallergens except musk xylene were correctly identified. Musk xylene is reported to have a weak photoallergenic effect (SCCNFP, 2004) and prohibited by IFRA from the viewpoint of not photosafety but VPVB (very persistent and very bio-accumulative) (IFRA, 2019). The ROS/mROS assay did not yield false-negative results because photoallergic chemicals bind to proteins to form hapten-protein conjugate via generation of ROS (Tokura, 2018), ROS assay theoretically captures all

photochemically active substances (Onoue and Tsuda, 2006), and some photolabile substances would be judged as positive if there are potent ROS generators in their photodegradation pathways (Onoue et al., 2016). In addition, superoxide anion can be measured upon the reduction of nitroblue tetrazolium, and the determination of singlet oxygen can be made on the basis of bleaching of *p*-nitrosodimethylaniline by oxidized imidazole (OECD, 2019b), and thus reducing or oxidizing chemicals (such as anti-oxidants) that interfere with these reactions tend to produce false-positive results. Our results showed that both the UV-VIS spectral analysis and ROS/mROS assay are useful for screening assessments while further assessment would be required to reduce false-positive predictions. Thus, this study aimed to develop an *in chemico* photoallergy testing method, photo-DPRA, as one of the follow-up tools.

In photo-DPRA for 34 positive controls, 30 chemicals were successfully detected, 2 chemicals (isoniazid and sulfasalazine) showed false-negative results and 2 chemicals (lomefloxacin and tetracycline) were outside of applicability due to coelution with cyst peptides. For 16 negative controls, 11 chemicals were successfully evaluated, 2 chemicals (methyl salicylate and penicillin G) showed false-positive results and 3 chemicals (aspirin, benzocaine and sulisbenzone) were outside of applicability. Isoniazid is an antibacterial agent used primarily as a tuberculostatic. Isoniazid is acetylated by N-acetyl transferase to N-acetylisoniazid; it is then biotransformed to isonicotinic acid and acetylhydrazine (Wishart et al., 2018). Acetylhydrazine was reported to bind proteins (Timbrell et al., 1980). Sulfasalazine is a drug that is used in the management of inflammatory bowel diseases and its activity is generally considered to lie in its metabolic breakdown component, 5-aminosalicylic acid (Wishart et al., 2018). 5-Aminosalicylic acid (also known as mesalazine, an anti-inflammatory agent) is reported to be a photoallergen (Onoue et al., 2017). These two chemicals are

considered to be pro-haptens which require metabolic activation to become photoallergens. Possibly other available information or testing methods addressing other key events of the photoallergy AOP need to be explored and integrated in the assessment strategy.

Fig. S1 gives an example of photo protein binding between cysteine peptide and the photoallergen. The reaction mixtures with and without cysteine peptide and/or irradiation using ketoprofen were prepared, and adduct formation was confirmed by the liquid chromatography-mass spectrometry. The result supported the usefulness of photo-DPRA for photoallergy assessments.

The SCCS notes of guidance stated that *“Presently, no validated in vitro methods for the detection of photo-sensitisation are available. Nevertheless, it is expected that chemicals showing photo-allergic properties are likely to give positive reactions in the 3T3 NRU PT test.”* (SCCS, 2021). Actually, many photoallergens were detected while the 3T3 NRU PT was not originally designed for assessing chemical photoallergy (Onoue et al., 2016). The action spectrum (the provocative light wavelength) of photoallergy is mostly ultraviolet A light (UVA; 320-400 nm), while ultraviolet B light (UVB; 290-320 nm) rarely evokes diseases (Tokura, 2018). This is in accordance with our UV-VIS spectral analysis results as shown in Table 4. The author also mentioned that all photoallergic chemicals have a phototoxic property because the photoallergic reaction requires the initial phototoxic step in which photoallergens bind to proteins via the formation of ROS. However, the sensitivity of 3T3 NRU PT was relatively low (72.7%) compared with that of others (UV-VIS spectral analysis, ROS/mROS assay, photo-DPRA) probably due to the UV absorbing range and solubility. A solar simulator with a filter to attenuate highly cytotoxic UVB is typically used, and thus the 3T3 NRU PT may give false-negative predictions for chemicals predominantly absorbing in the UVB range

(Ceridono et al., 2012). In addition, some photoallergens may be poorly soluble in an aqueous medium, possibly providing false-negative predictions. Therefore, the 3T3 NRU PT might be less effective for assessing photoallergy compared with photo-DPRA.

As for positive control, 34 chemicals listed in Table 1 were used in this study. In the field of skin sensitization, a murine Local Lymph Node Assay (LLNA) (OECD, 2010) is regarded as a gold standard method and the LLNA datasets were compiled for the purpose of non-animal methods development. In contrast, no validated *in vivo* photoallergy methods and gold standard positive control database exist. Therefore, clinical characteristics and pathogenetic mechanisms of chemical photoallergy were reviewed and most photoallergens were compiled on a clinical basis (Oncue et al., 2017). This photoallergy database might need further investigation and scrutiny for developing reliable *in chemico/in vitro/in silico* photoallergy tests.

Several *in chemico* or *in vitro* photoallergy methods have been reported so far, but none of them have yet to be validated and regulatory accepted. As for methods focused on photoantigen binding formation, human serum albumin is utilized by measuring increased UV absorbance of the protein fraction after gel filtration chromatography (Lovell and Jones, 2000), and the THP-1 cell line is utilized by monitoring changes in cell-surface thiols and amines (Oeda et al., 2016). Recently, photo-DPRA has been reported with the introduction of the UVA irradiation step (de Ávila et al., 2017; Patel et al., 2019); however, de Ávila et al. (2017) only focused on agrochemical compounds, and Patel et al. (2019) assessed only six phototoxic chemicals used as NRU 3T3 PT proficiency chemicals. Very recently, photo-amino acid derivative reactivity assay (ADRA), in which the test compound concentration was 1/100 compared to DPRA, was developed (Yamamoto et al., 2020), and it has some advantages that solubility and co-elution issues are less occurred. In

contrast, DPRA is a most widely used *in chemico* skin sensitization method and cost-effective. For chemicals that are outside of photo-DPRA applicability domain in terms of water solubility and co-elution, photo-ADRA could be used instead.

In this study, the experimental conditions of the photo-DPRA were optimized. Chemicals with a wide structural diversity were assessed and the results showed the utility of photo-DPRA to support the distinction between photoallergens and non-photoallergens. In addition, an *in chemico* STS consisting of the UV-VIS spectral analysis, ROS/mROS assay and photo-DPRA exhibited high prediction performance for assessing photoallergic potential. The data presented here will be used for a reference database when developing complete AOP-based IATA approaches.

Conflict of interest

The authors did not report any conflict of interest.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

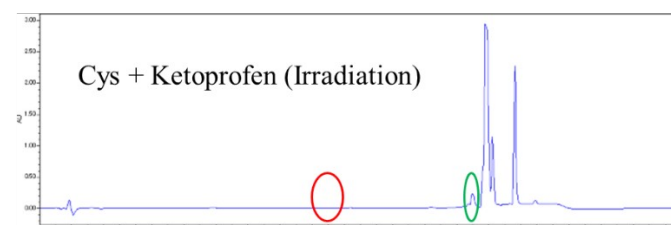
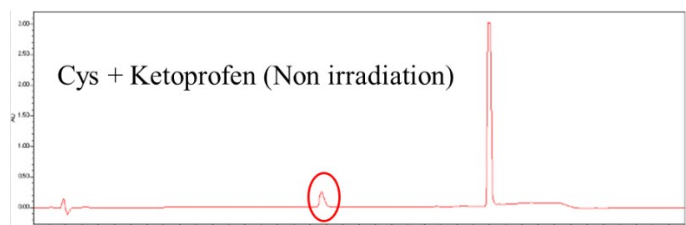
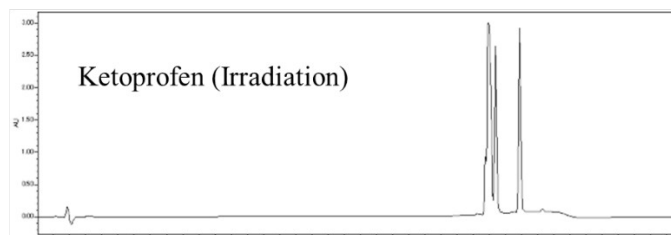
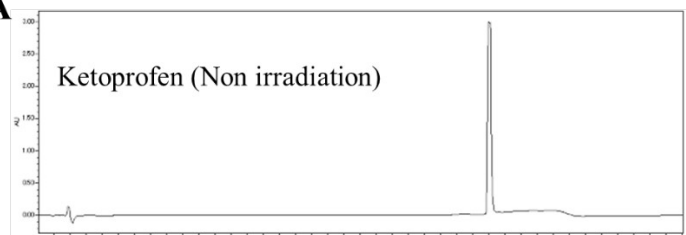
☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Journal Pre-proof

Highlights (20 words/bullet point)

- The UV-VIS spectral analysis and ROS/mROS assay showed high sensitivity but low specificity for assessing photosensitization.
- Photo-DPRA was developed based on original DPRA (OECD TG442C) method with addition of UV-VIS light irradiation as one of follow-up assessments.
- The proposed *in chemico* sequential testing strategy enables to assess the photosensitization potential of chemicals with high accuracy.

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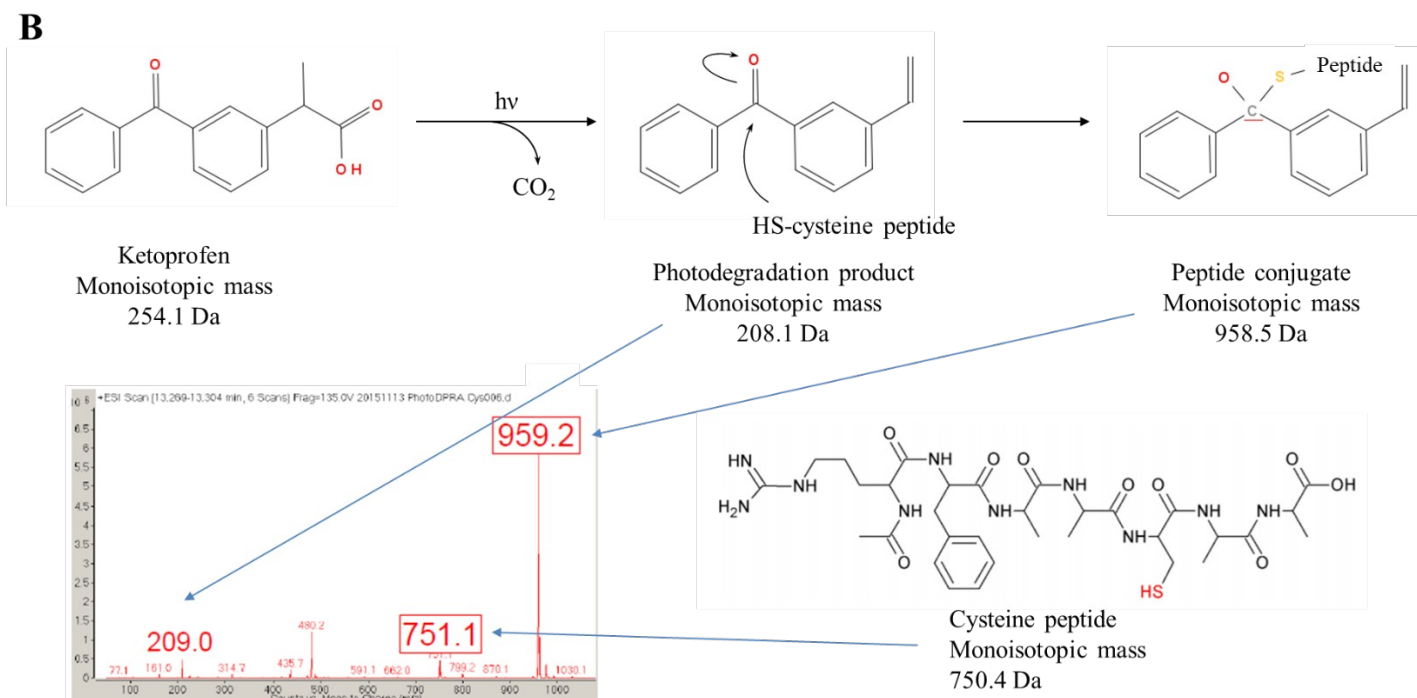


Fig. S1 Confirmation of photo protein binding between cysteine peptide and ketoprofen by LC/MS (Agilent UPLC system coupled with Agilent 6430

Triple quad LC/MS).

(A) Total ion chromatogram of the reaction mixtures with and without cysteine peptide and/or irradiation. Cysteine peptide was depleted only when irradiated as shown in red circle, while a new peak was detected only when irradiated as shown in green circle.

(B) Estimated formation of hapten-peptide conjugate and the mass spectrum. The m/z 959.2 indicates the predominant protonated ion $[\text{M} + \text{H}]^+$ of the

peptide conjugate. The two fragment ions (m/z 209.0 and 751.1) are probably derived from ketoprofen photodegradation product and cysteine peptide, respectively.