

Development of novel *in vitro* photosafety assays focused on the Keap1–Nrf2–ARE pathway

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ABSTRACT: Although photoallergens require UV energy for antigen formation, the subsequent immune response is considered to be the same as in ordinary skin sensitization. Therefore, *in vitro* tests for skin sensitization should also be applicable for photoallergy testing. In this study, we examined whether activation of the Keap1 (Kelch-like ECH-associated protein 1)–Nrf2 (nuclear factor-erythroid 2-related factor 2)–ARE (antioxidant response element) pathway could be used to assess the photoallergenic potential of chemicals, using the reporter cell line AREc32 or KeratinoSensTM. First, we identified an appropriate UVA irradiation dose [5 J cm⁻² irradiation in phosphate-buffered saline (PBS)] by investigating the effect of UV irradiation on ARE-dependent gene induction using untreated or 6-methylcoumarin (6-MC)-treated cells. Irradiation of well-known photoallergens under this condition increased ARE-dependent gene expression by more than 50% compared with both vehicle and non-irradiated controls. When the cut-off value for detecting photoallergens was set at 50% induction, the accuracy of predicting photoallergenic/phototoxic chemicals was 70% in AREc32 cells and 67% in KeratinoSensTM cells, and the specificity was 100% in each case. We designate these assays as a photo-ARE assay and photo-KeratinoSensTM, respectively. Our results suggest that activation of the Keap1–Nrf2–ARE pathway is an effective biomarker for evaluating both photoallergenic and phototoxic potentials. Either of the above tests might be a useful component of a battery of *in vitro* tests/*in silico* methods for predicting the photoallergenicity and phototoxicity of chemicals. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: photoallergy; *in vitro*; Keap1; Nrf; ARE; KeratinoSensTM

Introduction

The safety evaluation of ingredients is a key step in the development of cosmetics and drugs. Potential hazards include phototoxicity and photoallergy, which are topically or systemically induced by exposure of skin to solar light in the presence of photoreactive compounds (generally referred to as photosensitizers). Such photoreactions occur mostly in response to ultraviolet A (UVA; 400–315 nm) light rather than the shorter-wavelength UVB and UVC components (Tokura, 2009). Phototoxicity is an acute toxic reaction that occurs upon exposure to UV even when a phototoxic compound is encountered for the first time (Gould *et al.*, 1995). The trigger of this response is reactive oxygen species (ROS), especially singlet oxygen, which are generated upon absorbance of UV light energy by the photosensitizer (Inbaraj *et al.*, 2005; Onoue and Tsuda, 2006); the resulting ROS damage cellular macromolecules and organelles (Gould *et al.*, 1995). In contrast, photoallergy does not occur upon first exposure to a photoallergen, because it is a consequence of immunological responses, i.e. the UV-induced photoantigen triggers T-cell responses. Various antibacterial agents (e.g. 3,3',4',5'-tetrachlorosalicylanilide), fragrance ingredients (e.g. 6-methylcoumarin) and anti-inflammatory drugs (e.g. ketoprofen) are photoallergens.

The phototoxicological properties of chemicals have generally been assessed in animals, such as guinea pigs, rabbits, rats and mice (Maurer *et al.*, 1980; Ichikawa *et al.*, 1981; Jordan, 1982; Gerberick and Ryan, 1990; Ulrich *et al.*, 1998). However, as a result of regulatory constraints and ethical concerns, the development of

alternative *in vitro* assays is urgently necessary. For phototoxicity testing, the *in vitro* 3T3 NRU phototoxicity test has already been adopted as an alternative test method (OECD, 2004). Recently, the ROS assay, which was developed for photosafety evaluation, was adopted by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) S10 guideline (Onoue *et al.*, 2013a; ICH S10, 2013). As for photoallergy, several *in vitro* methods have been proposed (Tokura, 2000; Lovell and Jones, 2000; Onoue and Tsuda, 2006; Kurita *et al.*, 2007; Hoya *et al.*, 2009; Karschuk *et al.*, 2010; Galbiati *et al.*, 2013), but none of them has yet been accepted for the prediction of the photoallergenic potential of new compounds.

Photoallergy is believed to be a cell-mediated delayed-type hypersensitivity reaction similar to contact allergy, differing only in that the hapten is a photosensitizer that requires radiant energy to become a photoantigen. In the induction phase of skin

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sensitization, haptens penetrate through the stratum corneum and activate antigen-presenting cells (APCs), including cutaneous dendritic cells (DCs). The activated DCs migrate into lymph nodes and present antigens to T cells. Great efforts have been made to develop an *in vitro* skin sensitization assay, focusing on Adverse Outcome Pathway (AOP) key events, such as protein reactivity and the activation of keratinocytes and DCs. It is recognized that no single *in vitro* method will be sufficient to predict the sensitizer potency because of the complexity of the underlying immune responses, and instead a combination of several methods is likely to be needed for skin sensitization assessment without using animal tests (Jowsey *et al.*, 2006). There have been several reports on the prediction of the skin sensitization potency of chemicals using *in vitro/in silico* results (Natsch *et al.*, 2009; Jaworska *et al.*, 2011; Nukada *et al.*, 2013; Tsujita-Inoue *et al.*, 2014, 2015), and a promising approach for predicting photoallergenic potency as well as skin sensitization would be to integrate methods that focus on different key events.

As an *in vitro* method focusing on photoantigen formation, photo-induced binding of test chemicals to human serum albumin has been utilized to assess the photoallergenic potential of chemicals (Lovell and Jones, 2000). As for *in vitro* methods focusing on activation of DCs, several groups, including ours, have reported that measurements of cell-surface molecules such as CD86, CD54 and/or HLA-DR on THP-1 (human monocytic leukemia cell line) or human peripheral blood monocyte-derived DCs can be used to identify photoallergens (Hoya *et al.*, 2009; Hino *et al.*, 2008; Karschuk *et al.*, 2010). In contrast, there have been few studies focusing on the mechanisms of keratinocyte activation, such as the Keap1 (Kelch-like ECH-associated protein 1)–Nrf2 (nuclear factor-erythroid 2-related factor 2)–ARE (antioxidant response element) regulatory pathway, which is deeply involved in skin sensitization.

Natsch and Emter (2008) reported that a majority of skin sensitizers induce genes regulated by ARE, and they developed a cell-based assay called the ARE assay using a reporter cell line, AREc32, which contains an eight-fold repeat of the rat GSTA2 ARE-sequence upstream of a luciferase reporter gene in human breast cancer cell line MCF7 (Natsch and Emter, 2008). The mechanism by which this pathway is activated by skin sensitizers is considered as follows. The sensor protein Keap1 contains highly reactive Cys residues, and small electrophilic substances such as skin sensitizers can act on Keap1 by covalent modification of its Cys residue. As a result, Keap1 dissociates from Nrf2 and the dissociated Nrf2 activates genes having ARE in their promoter sequence (Wakabayashi *et al.*, 2004; Dinkova-Kostova *et al.*, 2005). They further developed an improved method called KeratinoSensTM, which more closely mimics the conditions under which skin sensitization occurs in human skin (Emter *et al.*, 2010). KeratinoSensTM employs HaCaT keratinocytes with the stable insertion of a luciferase reporter gene under the control of a single copy of ARE of human AKR1C2. These two methods are both cited as references for the Key Event 2 of the AOP for skin sensitization (OECD, 2012). The accuracy of the ARE assay and KeratinoSensTM was 83.0% and 77.1%, the sensitivity was 81.4% and 78.0%, and the specificity was 86.6% and 76.4%, respectively (Natsch and Emter, 2008; OECD, 2015). In February 2015, KeratinoSensTM was adopted by the OECD as test guideline 442D for skin sensitization.

In this study, we evaluated whether activation of the Keap1–Nrf2–ARE a regulatory pathway, which is deeply involved in the induction phase of skin sensitization as described above, is available for identification of photoallergens.

Materials and methods

Chemicals

The majority of the test chemicals were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA). Sources of other chemicals were as follows: 3,3',4',5-tetrachlorosalicylanilide (TCSA), Acros Organics (Morris Plains, NJ, USA); 6-methylcoumarin (6-MC) and 3,4',5-tribromosalicylanilide, Tokyo Chemical Industry (Tokyo, Japan); chlorpromazine HCl and sulfanilamide, Nacalai tesque (Kyoto, Japan); sodium lauryl sulfate, anthracene, L(+)-ascorbic acid and dextran, Wako Pure Chemicals (Osaka, Japan); isoniazid, LKT Laboratories (St. Paul, MN, USA); acridine, Lancaster Synthesis (Ward Hill, MA, USA); diclofenac sodium salt, Cayman Chemical (Ann Arbor, MI, USA); benzydamine HCl, MP Biomedicals (Santa Ana, CA, USA); musk ambrette was from a fragrance company.

UV light source

A SOL500 Sun Simulator (Dr Hönle AG, Grägelting, Germany) equipped with a 500-W metal halide lamp and an H1 filter to attenuate UVB was used. UVA-Meters (Dr Hönle AG for photo-ARE assay and Topcon UVR-3036/S2 for photo-KeratinoSensTM) were used to measure the UVA intensity.

Cell culture

AREc32 is a stable cell line derived from the human MCF7 breast carcinoma cell line (Natsch and Emter, 2008). AREc32 cells were obtained from CXR Biosciences, Dundee, Scotland. AREc32 cells were maintained in Dulbecco's modified Eagle's medium containing Glutamax supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin (all from Gibco, CA, USA) and 500 µg ml⁻¹ G418 (Calbiochem, CA, USA) in an incubator under an atmosphere of 5% CO₂ in air.

KeratinoSensTM is a stable cell line derived from HaCaT human keratinocytes (OECD, 2015). KeratinoSensTM cells were obtained from Givaudan, Vernier, Switzerland. KeratinoSensTM cells were maintained in Dulbecco's modified Eagle's medium containing Glutamax supplemented with 9% (v/v) FBS and 500 µg ml⁻¹ G418 (Calbiochem, CA, USA) in an incubator under an atmosphere of 5% CO₂ in air. Cells until passage 25 were used for the assay.

Pre-study cytotoxicity assay

AREc32 cells were seeded in 96-well plates at a density of 50 000 cells per well in 180 µl of growth medium and pre-cultured for 48 h. The test chemicals were dissolved in phosphate-buffered saline (PBS) at the highest concentration of 100 mg ml⁻¹ for water-soluble chemicals or in dimethyl sulfoxide (DMSO) at the highest concentration of 500 mg ml⁻¹ for water-insoluble chemicals, and then six binary dilutions were made. If DMSO was used as a solvent, the solutions were further diluted 10-fold with PBS so that the final concentration of DMSO was less than 1%. After the pre-culture period, the medium was replaced with 180 µl of PBS and cells were treated with 20 µl of various concentrations of chemicals for 1 h and then irradiated with UV. Control samples were kept in the dark under the same conditions. After UV irradiation, PBS was replaced with fresh cell culture medium and the cells were cultured in a CO₂ incubator for 24 h.

Cells were washed twice in PBS without Ca²⁺ and Mg²⁺ [PBS(-)], and cell viability was determined using CellTiter-Glo Luminescent

Cell Viability Assay Reagent with a GloMax Luminometer (Promega KK, Tokyo, Japan). The concentration providing a cell viability of 75% (CV75) was calculated for each chemical by interpolation on the log-linear dose-response curve.

Photo-ARE assay procedure

AREc32 cells were seeded and treated with chemicals in the same way as described for the cytotoxicity assay. The test doses were set at $1/8 \times \text{CV75}$, $1/4 \times \text{CV75}$, $1/2 \times \text{CV75}$, CV75, $2 \times \text{CV75}$ and $4 \times \text{CV75}$ as the final concentrations. If the CV75 could not be determined, the test doses were set at six binary dilutions from the maximum concentration of $10\,000\ \mu\text{g ml}^{-1}$ (for water-soluble chemicals) or $5000\ \mu\text{g ml}^{-1}$ (for water-insoluble chemicals) or from the concentration corresponding to the maximum solubility in each solvent.

UV irradiation and subsequent medium exchange were carried out as in the cytotoxicity assay. At 24 h after UV irradiation, cells were washed twice with PBS(-) and ARE-dependent gene expression was measured using a Steady-Glo Luciferase Assay System (Promega KK, Tokyo, Japan) with the Glomax luminometer. Cytotoxicity of the compounds to AREc32 cells was tested in parallel assays run under the same conditions and with the same test concentrations as described above.

Data analysis for photo-ARE assay

The assay was repeated three times with duplicate analyzes for each chemical at each test concentration in each repetition. A chemical was rated positive if the gene activity was enhanced more than 1.5 times compared with the vehicle control at any of the test concentrations when UV exposure was carried out, and if, in addition, at the same test concentrations, the gene activity was induced more than 1.5 times compared with the non-irradiated control either in all repetitions or two out of three repetitions. It was also required that there should be an apparent overall dose-response relationship. When the cell viability was less than 50%, the relative luciferase activity was not used in the determination.

Photo-KeratinSens™ procedure

KeratinSens™ cells were seeded in 96-well plates at a density of 30 000 cells per well in 125 μl of growth medium without G418 and pre-cultured for 24 h. The test chemicals were dissolved in DMSO at a concentration of 200 mM and were serially diluted in DMSO to obtain 12 final concentrations ranging from 0.1 to 200 mM. These DMSO solutions were diluted 25-fold in PBS. After the pre-culture period, the medium was replaced with 150 μl of

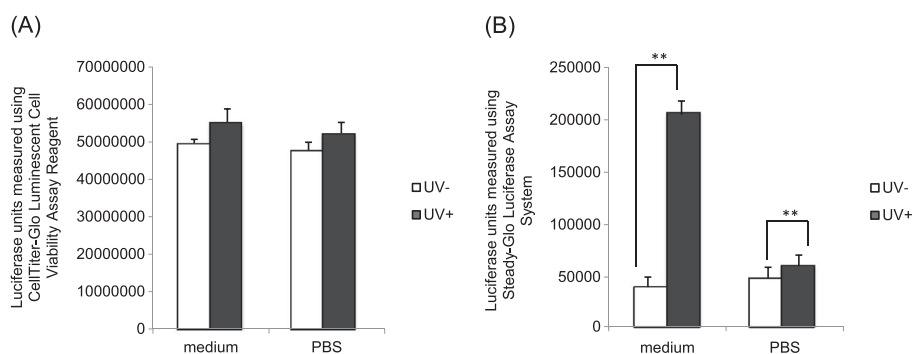


Figure 1. Effects of UV irradiation on (A) cell viability and (B) ARE-dependent gene expression of AREc32. Luciferase activity was measured with (A) CellTiter-Glo Luminescent Cell Viability Assay Reagent or (B) the Steady-Glo Luciferase Assay System. Cells were irradiated at the dose of $5\ \text{J cm}^{-2}$ in cell culture medium or phosphate-buffered saline (PBS). The results were expressed as the mean $\pm$ SD for at least three independent experiments. Statistical analysis was performed with *t*-test. $**P < 0.01$.

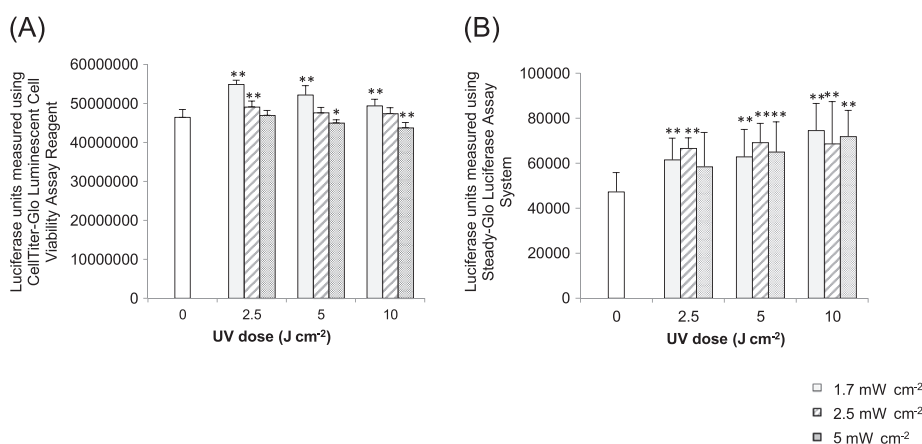


Figure 2. Effects of irradiation intensity and dose on (A) cell viability and (B) ARE-dependent gene expression of untreated cells. Cells were irradiated with a UV dose of 2.5, 5 or $10\ \text{J cm}^{-2}$ at the intensity of 1.7, 2.5 or $5\ \text{mW cm}^{-2}$, and then incubated for 24 h at 37°C . Luciferase activity was measured with (A) CellTiter-Glo Luminescent Cell Viability Assay Reagent or (B) the Steady-Glo Luciferase Assay System. The results were expressed as the mean $\pm$ SD of at least three independent experiments. Statistical analysis was performed with Dunnett's test. $*P < 0.05$, $**P < 0.01$.

PBS and cells were treated with 50 μ l of various concentrations of chemicals for 1 h and then irradiated with UV. Control samples were kept in the dark under the same conditions. After UV irradiation, PBS was replaced with fresh cell culture medium containing 1% FBS and cells were cultured in a CO₂ incubator for 48 h. Cell viability and ARE-dependent gene expression were measured as described above.

Data analysis for Photo-KeratoSens™

Data analysis was done in the same manner as in photo-ARE assay. When the cell viability was less than 70%, the relative luciferase activity was not used in the determination, according to the original protocol of KeratoSens™.

Results

Establishment of irradiation conditions for photo-ARE assay

Study on medium for UV irradiation. First, we examined whether culture medium components such as pH indicator, vitamins or serum proteins influence cell viability or ARE-dependent gene expression under UV irradiation. After the pre-culture period, the culture medium was replaced with PBS or kept unchanged, and the plate was exposed to UVA (5 J cm⁻² at the intensity of 1.7 mW cm⁻²; determined by reference to the irradiation conditions of the 3T3 NRU phototoxicity test) (OECD, 2004). For cells subjected to UV exposure in PBS, medium change to the cell culture

medium was performed after UV irradiation. At 24 h later, cell viability and ARE-dependent gene expression were measured using luciferase activity as the indicator. As shown in Fig. 1A, there was no significant change in cell viability in response to the UV exposure between cultures in medium and in PBS. In contrast, Fig. 1B shows that UV exposure increased ARE-dependent gene expression with statistical significance. The ARE-dependent gene expression was more than five times greater in cells irradiated in the medium than those non-irradiated. It was difficult to assess the chemical enhancement of ARE-dependent gene expression when the expression was markedly enhanced by UV irradiation alone. Therefore, we decided to treat cells with chemicals in PBS during UV treatment, as only minimal enhancement of ARE-dependent gene expression by UV irradiation was seen in PBS.

Study of UV dose and intensity in the photo-ARE assay. Next, we assessed the effects of three irradiation protocols (measured UV intensities of 1.7, 2.5 and 5 mW cm⁻²). As shown in Fig. 2, luciferase was weakly induced by CellTiter-Glo or Steady-Glo reagents. As the maximum induction was less than 1.3 times, it was considered that this would not affect the evaluation of the effect of test chemicals.

To establish the optimal irradiation dose in the presence of a photoallergen, 6-methylcoumarin (6-MC) was used as a representative photoallergen. The dose setting of 6-MC was started from 250 μ g ml⁻¹, as this concentration was not cytotoxic to non-irradiated cells. As shown in Fig. 3A, a UV irradiation dose of 2.5 J cm⁻² caused slight enhancement of ARE-dependent gene

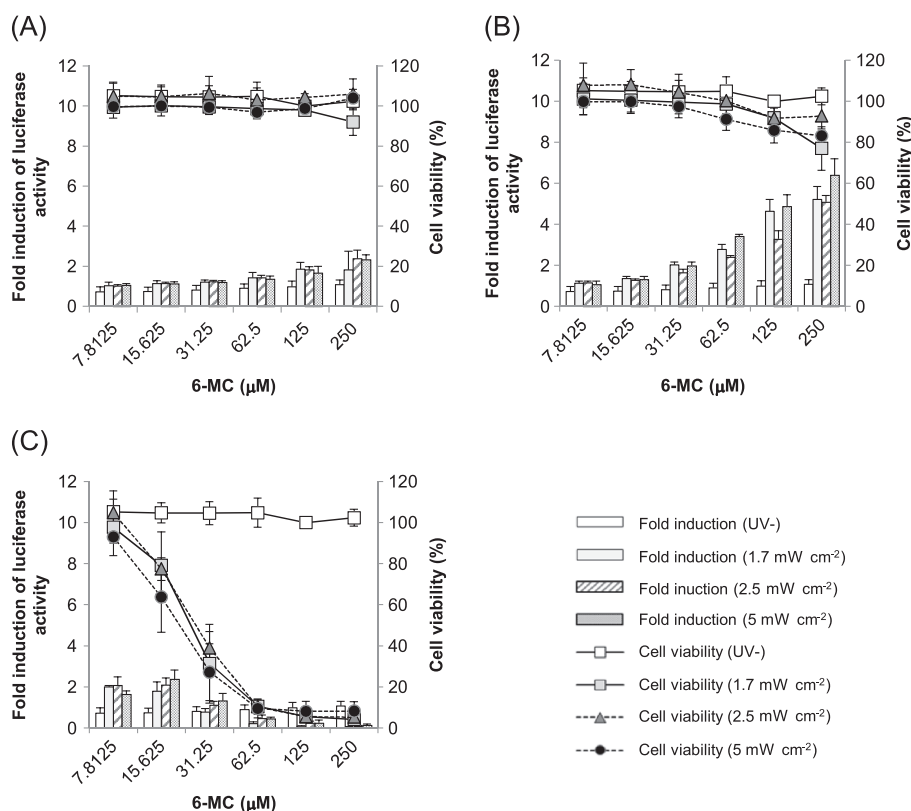


Figure 3. Effects of UV intensity and the total amount of irradiation on ARE-dependent gene expression and viability of 6-MC-treated AREc32 cells. Cells were irradiated at various intensities and dose levels [(A) 2.5 J cm⁻², (B) 5 J cm⁻² and (C) 10 J cm⁻²] as shown, after treatment with various concentrations of 6-MC. After irradiation, cells were incubated for 24 h at 37 °C. Induction (fold) of luciferase activity and cell viability were calculated. The results were expressed as the mean $\pm$ SD of at least three independent experiments.

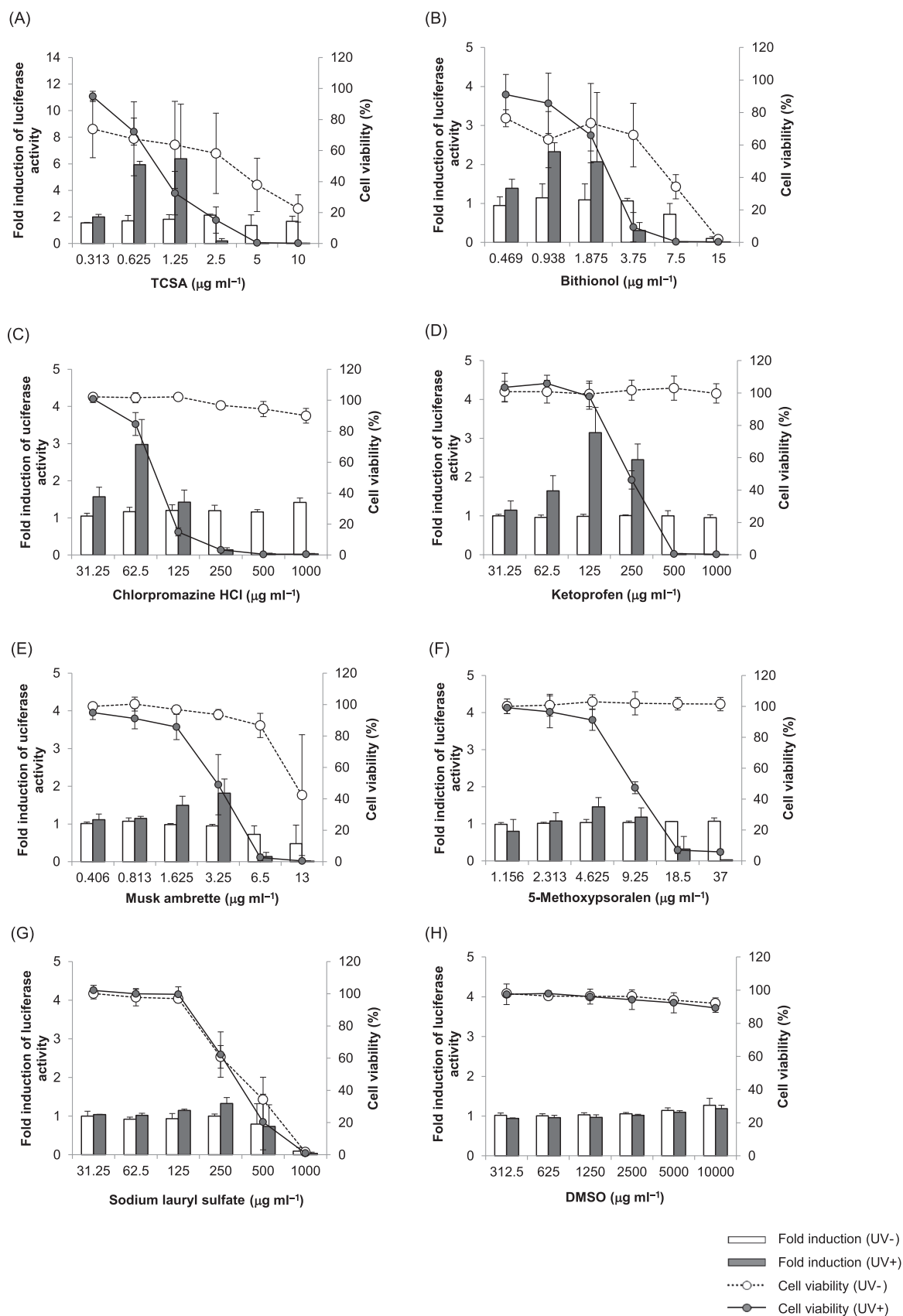


Figure 4. Effects of chemicals, together with 5 J cm⁻² UV irradiation, on luciferase activity and cell viability. (A) 3,3',4',5-tetrachlorosalicylanilide (TCSA), (B) bithionol, (C) chlorpromazine HCl, (D) ketoprofen, (E) musk ambrette, (F) 5-methoxypsoralen, (G) sodium lauryl sulfate and (H) dimethyl sulfoxide (DMSO). Cells were irradiated at 5 J cm⁻² UV at 1.7 mW cm⁻² after treatment with the test chemical. After irradiation, cells were further incubated for 24 h at 37 °C. Induction (fold) of luciferase activity and cell viability were calculated. Results are expressed as the mean ± SD of at least three independent experiments.

Table 1. Chemical information and results of photo-ARE assay of 30 chemicals.

No	Test chemicals	CAS No.	in vivo or clinical data		Results of photo ARE assay					Solvent	Reference	
					photoallergic	phototoxic	Judgement	N ^a	Imax ^b (UV+/UV-)			Cmax ^c (µg/ml)
Photoallergenic/phototoxic chemicals												
1	6-Methylcoumarin	92-48-8	+	+	P	3/3	4.52	250	4.92	1.11	DMSO	Lovell and Jones (2000)
2	3, 3', 4', 5-Tetrachlorosalicylanilide	1154-59-2	+	+	P	2/3	4.03	0.625	5.99	1.55	DMSO	Nishijima et al. (1999)
3	Bithionol	97-18-7	+	+	P	2/3	3.19	1.875	2.10	0.93	DMSO	Lovell and Jones (2000)
4	Chlorpromazine HCl	69-09-0	+	+	P	3/3	2.55	0.9375	2.98	1.17	DMSO	Lovell and Jones (2000)
5	Prometazine HCl	58-33-3	+	+	P	3/3	2.35	3.75	3.49	1.48	DMSO	Lovell and Jones (2000)
6	3,4',5-tribromosalicylanilide	87-10-5	+	+	P	3/3	5.57	1.875	8.25	1.48	DMSO	Onoue et al. (2013b)
7	Diclofenac sodium salt	15307-79-6	+	+	P	3/3	2.13	210	2.71	1.25	DMSO	Onoue et al. (2013b)
8	Ketoprofen	22071-15-4	+	-	P	3/3	3.19	6.25	3.14	0.99	DMSO	Lovell and Jones (2000)
9	Musk ambrette	83-66-9	+	-	P	2/3	1.68	3.25	1.62	0.96	DMSO	Onoue et al. (2013b)
10	p-Phenylenediamine	106-50-3	+	+	P	2/3	1.71	156.25	5.48	3.20	DMSO	LeVine (1984), National Toxicology
11	Piroxicam	36322-90-4	+	+	N	0/3	0.97	500	0.87	0.89	DMSO	Hariya et al. (1993)
12	Enoxacin	74011-58-8	+	+	P	3/3	2.42	13.75	2.52	1.04	DMSO	Tokura (1998)
13	Sulfanilamide	63-74-1	+	+	N	0/3	1.06	2500	1.12	1.06	DMSO	Onoue et al. (2013b)
14	Trichlorocharban	101-20-2	+	+	N	0/3	1.09	0.46875	1.60	1.41	DMSO	Onoue et al. (2013b)
15	Benzophenone	119-61-9	+	+	P	3/3	3.26	9.25	3.18	0.98	DMSO	Landers et al. (2003)
16	Benzylamine	132-69-4	+	+	N	1/3	1.48	157.5	1.65	1.11	PBS	Tokura (2009)
17	Tenoxicam	59804-37-4	+	+	N	1/3	1.44	1000	2.11	1.45	DMSO	Hariya et al. (1993)
18	5-Methoxypsoralen	484-20-8	-	+	N	1/3	1.40	0.0375	1.46	1.04	DMSO	Lovell and Jones (2000)
19	Antracene	120-12-7	-	+	P	3/3	1.86	0.0125	1.98	1.08	DMSO	Lovell and Jones (2000)
20	Acridine	260-94-6	-	+	N	1/3	1.53	0.25	1.79	1.17	DMSO	Lovell and Jones (2000)
21	Furosemide	54-31-9	+	+	N	1/3	1.51	500	1.78	1.18	DMSO	Vargas et al. (1998)
22	Amiodarone HCl	19774-82-4	+	+	P	3/3	1.78	3.75	1.89	1.06	DMSO	Onoue et al. (2013b)
23	Indomethacin	53-86-1	+	+	N	0/3	1.29	87.5	1.26	0.97	DMSO	Onoue et al. (2013b)
Non-phototoxic chemicals												
24	Sodium lauryl sulfate	151-21-3	-	-	N	0/3	0.94	3.125	1.04	1.00	PBS	Lovell and Jones (2000)
25	Lactic Acid	50-21-5	-	-	N	0/3	0.97	750	1.08	1.11	PBS	Onoue et al. (2013b)
26	Methylsalicylate	119-36-8	-	-	N	0/3	1.02	150	1.05	1.03	DMSO	Onoue et al. (2013b)
27	Penicilin G sodium salt	69-57-8	-	-	N	0/3	1.20	10000	1.75	1.47	PBS	Onoue et al. (2013b)
28	DMSO	67-68-5	-	-	N	0/3	0.96	2500	1.02	1.06	PBS	Onoue et al. (2013b)
29	Dextran	9004-54-0	-	-	N	0/3	0.94	1250	0.95	1.02	PBS	Onoue et al. (2013b)
30	L(+)-Ascorbic acid	50-81-7	-	-	N	0/3	1.23	337.5	1.36	1.10	PBS	Onoue et al. (2013b)

CAS No.; Chemical Abstract Service number. P; Positive, N; Negative.

^aN: Number of repetitions with significant luciferase induction/number of repetitions made.^bImax: The highest average value of fold induction of luciferase activity at each test concentration among the three repetitions.^cCmax: Concentration for Imax.^dAverage of fold induction at Cmax: The average value excluding data rejected because of cytotoxicity.

Table 2. Correlation between *in vivo* or clinical data for (A) photoallergenic/phototoxic chemicals and (B) photoallergens, and photo-ARE assay results.

		Photo ARE assay	
		Positive	Negative
(A) <i>in vivo</i> or clinical data	Photoallergenic/phototoxic chemicals	14	9
	Non-phototoxic chemicals	0	7
	Accuracy	70%	
	Sensitivity	61%	
	Specificity	100%	
(B) <i>in vivo</i> or clinical data	Photoallergenic chemicals	12	5
	Non-photoallergenic chemicals	2	11
	Accuracy	77%	
	Sensitivity	71%	
	Specificity	85%	

Grey shading indicates that the same results were obtained in *in vivo* or clinical data and in the photo ARE assay.

expression (luciferase induction) compared with the non-irradiated cells, and it had almost no effect on cell viability. Luciferase induction was enhanced significantly in a dose-dependent manner with UV irradiation of 5 J cm^{-2} (Fig. 3B). At a higher concentration of 6-MC, slight cytotoxic effects were observed, and induction of luciferase activity reached about five times, compared with non-irradiated cells. A UV irradiation dose of 10 J cm^{-2} caused severe cytotoxicity and relatively weak luciferase induction even at subtoxic doses of 6-MC (Fig. 3C). This level of UV irradiation had no marked luciferase-inducing effect or cytotoxicity.

The present data indicate that an irradiation dose of 5 J cm^{-2} is an appropriate condition. This irradiation dose is identical to that of the 3T3 NRU PT (OECD, 2004). We chose the irradiation intensity of 1.7 mW cm^{-2} , as these are the same conditions as used in our previously reported photosensitization assay (Hoya et al., 2009). We used these conditions in the following experiments.

Selective induction of luciferase activity in response to photoallergen after UV irradiation in photo-ARE assay

To evaluate whether our *in vitro* photosensitization assay can detect photoallergens under the above irradiation conditions, the following chemicals were selected and tested: TCSA, bithionol, chlorpromazine HCl, ketoprofen and musk ambrette as photoallergens, 5-methoxypsoralen as a phototoxic substance, and sodium lauryl sulfate and DMSO as non-phototoxic substances.

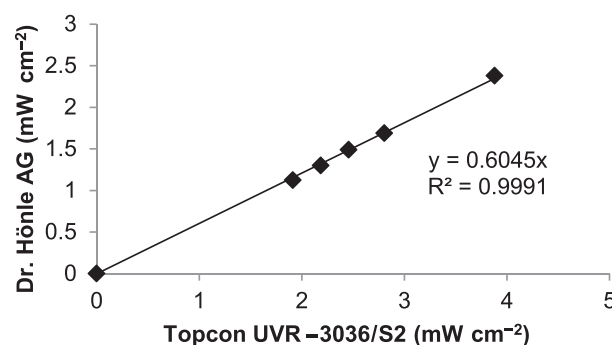
As shown in Fig. 4, the luciferase activity was enhanced by these photoallergens after UV irradiation. Given that the test material acts as a skin sensitizer, luciferase expression might be enhanced without UV irradiation, because the original ARE assay was reported as an *in vitro* skin sensitization assay. Therefore, the ratio of luciferase expression in UV-irradiated cells to non-irradiated cells should be an important parameter to evaluate photoallergenicity. The five photoallergens were judged as positive based on the criteria that luciferase induction after UV irradiation was more than 1.5 times greater than the vehicle control, and further that it was more than 1.5 times greater than the non-irradiated control.

Predictive accuracy, sensitivity and specificity of the *in vitro* photoallergy test

To set up appropriate criteria for identifying photoallergens, we evaluated 30 chemicals in the photo-ARE assay. The results are summarized in Table 1. Using the above criteria, 12 of 17 photoallergens and 2 of 6 phototoxic chemicals were judged as positive. All 7 non-phototoxic chemicals were judged as negative. As shown in Table 2, the values of predictive accuracy, sensitivity and specificity of the photo-ARE assay in relation to reported *in vivo* or clinical data on photoallergenic/phototoxic chemicals were 70%, 61% and 100%, respectively (Table 2A). When test substances were limited to photoallergens, the predictive accuracy, sensitivity and specificity were 77%, 71% and 85%, respectively (Table 2B).

Establishment of irradiation conditions of photo-KeratinSens™

First, we established suitable irradiation conditions for photo-KeratinSens™ in the same way as for the photo-ARE assay. In this study, we used a Topcon UVR-3036/S2 as UVA meter. We confirmed that there was a high correlation between UVA intensity

**Figure 5.** Correlation between the UV intensity measured with different UV meters (Topcon and Dr Höhle).

values determined with the Dr Hönle instrument used for photo-ARE assay and the Topcon UVR-3036/S2 (Fig. 5). When KeratinoSensTM cells were seeded in a 96-well plate at a density of 10 000 cells per well (the same as in the original protocol) and irradiated with 5 J cm^{-2} of UV in PBS in the presence of various concentrations of 6-MC, no significant induction of luciferase activity was observed compared with non-irradiated cells (Fig. 6A). As no

marked luciferase induction was observed with increasing doses of UV (data not shown), we examined the effect of cell concentration on the luciferase activity. When cells were seeded at a density of 30 000 cells per well, the luciferase activity was increased almost 3-fold, and cytotoxicity was reduced (Fig. 6B). Thus, we fixed the number of cells at 30 000 cells per well, and assessed the effects of three irradiation levels (measured UV intensities of 1.9, 2.5 and

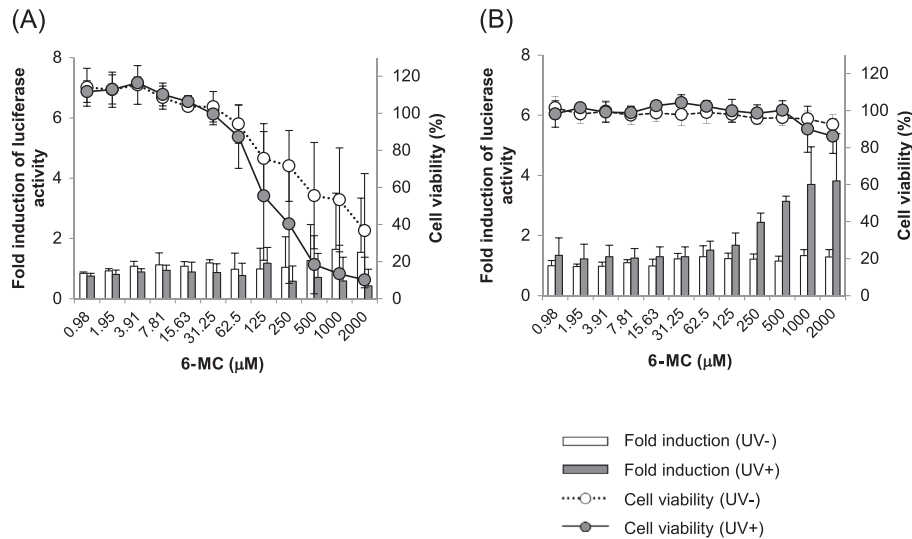


Figure 6. Effect of cell number on induction (fold) of luciferase activity and viability of cells treated with 6-MC together with 5 J cm^{-2} UV irradiation. KeratinoSensTM cells were seeded in 96-well plates at the density of (A) 10 000 cells per well or (B) 30 000 cells per well. The results are expressed as the mean $\pm$ SD of at least three independent experiments.

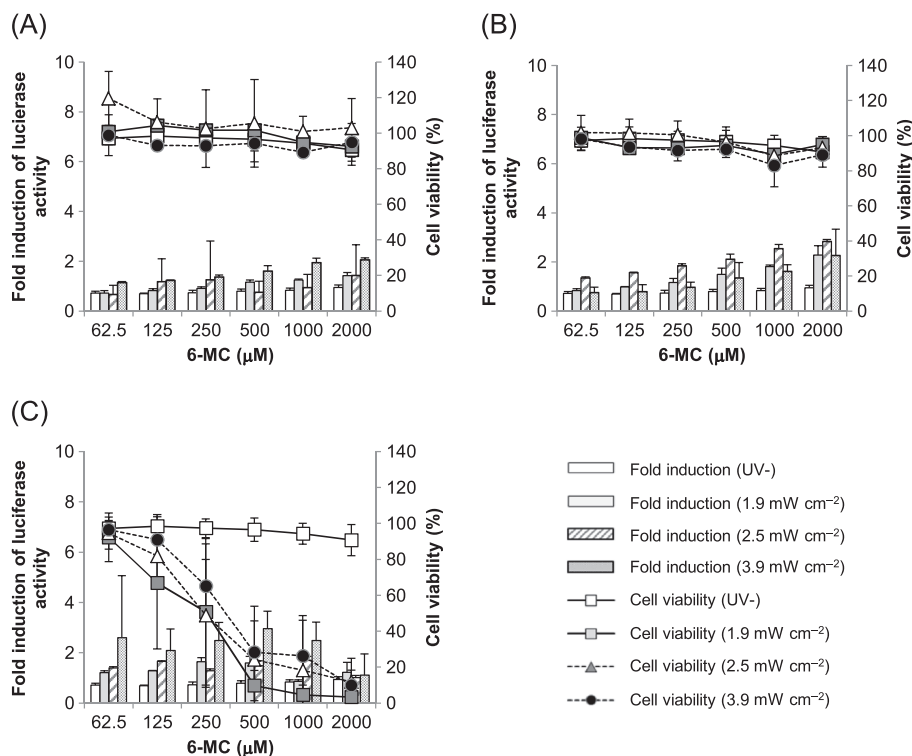


Figure 7. Effects of UV intensity and dose on luciferase induction by 6-MC and cell viability in KeratinoSensTM. Cells were irradiated at various intensities and doses [(A) 2.5 J cm^{-2} , (B) 5 J cm^{-2} and (C) 10 J cm^{-2}] after treatment with various concentrations of 6-MC. After irradiation, cells were incubated for 48 h at 37°C . Induction (fold) of luciferase activity and cell viability were calculated. The results are expressed as the mean $\pm$ SD of at least three independent experiments.

3.9 mW cm⁻²) by exposing 6-MC-treated cells to increasing UV doses. As shown in Fig. 7, UV irradiation at 2.5 and 5 J cm⁻² caused almost no cytotoxicity whereas luciferase expression was markedly enhanced by 5 J cm⁻² UV. In contrast, UV irradiation of 10 J cm⁻² caused severe cytotoxicity and relatively weak luciferase induction even at sub toxic doses of 6-MC. Thus, we concluded that a radiation dose of 5 J cm⁻² was appropriate. When 6-MC-treated cells were irradiated with 5 J cm⁻² UV, luciferase expression tended to be most enhanced by 2.5 mW cm⁻² intensity. Therefore, we chose the irradiation dose of 5 J cm⁻² at the intensity of 2.5 mW cm⁻² and used these conditions for all further experiments.

Selective enhancement of ARE-dependent gene expression in response to photoallergens after UV irradiation in photo-KeratinSensTM

We evaluated nine chemicals, which had already been evaluated in the photo-ARE assay, in photo-KeratinSensTM. The cellular responses to a range of common concentrations were compared because the way of determining the applied dose was different in the two assays. As shown in Fig. 8, while photo-KeratinSensTM tended to be less susceptible to cytotoxicity, luciferase induction was almost the same as in photo-ARE assay. Therefore, we used the same criteria as in photo-ARE assay to detect photoallergens, that is, luciferase induction after UV irradiation was more than 1.5 times those of the vehicle control and the non-irradiated control.

The results are summarized in Table 3. Among the nine evaluated chemicals, the final decision differed from the result of the photo-ARE assay only in the case of 5-MOP. For these photoallergenic/phototoxic chemicals, the accuracy, sensitivity and specificity of photo-KeratinSensTM versus *in vivo* or clinical data were 67%, 57% and 100%, i.e. almost the same as those of photo-ARE assay (Table 3A). When test substances were limited to photoallergens, the predictive accuracy, sensitivity and specificity were 56%, 50% and 67%, respectively (Table 4B). Evaluations of a range of other chemicals are in progress.

Discussion

In this study, we focused on the Keap1-Nrf2-ARE regulatory pathway as a key common step in the AOP for photoallergens. Activation of this pathway has already been utilized in the ARE assay using AREc32 cells derived from breast cancer (Natsch and Emter, 2008) and KeratinSensTM using cells derived from keratinocytes (Emter *et al.*, 2010) to detect skin sensitizers. Here, we examined the suitability of these methods for identifying photoallergens by adding a UV irradiation step. Initial studies indicated that some substance(s) in the culture medium activated the Keap1-Nrf2-ARE regulatory pathway in response to UV irradiation. This effect was not due to phenol red, which is contained in the medium as a pH indicator and is known to be photoreactive; we speculate it might be due to serum protein(s). In any event, the effect could

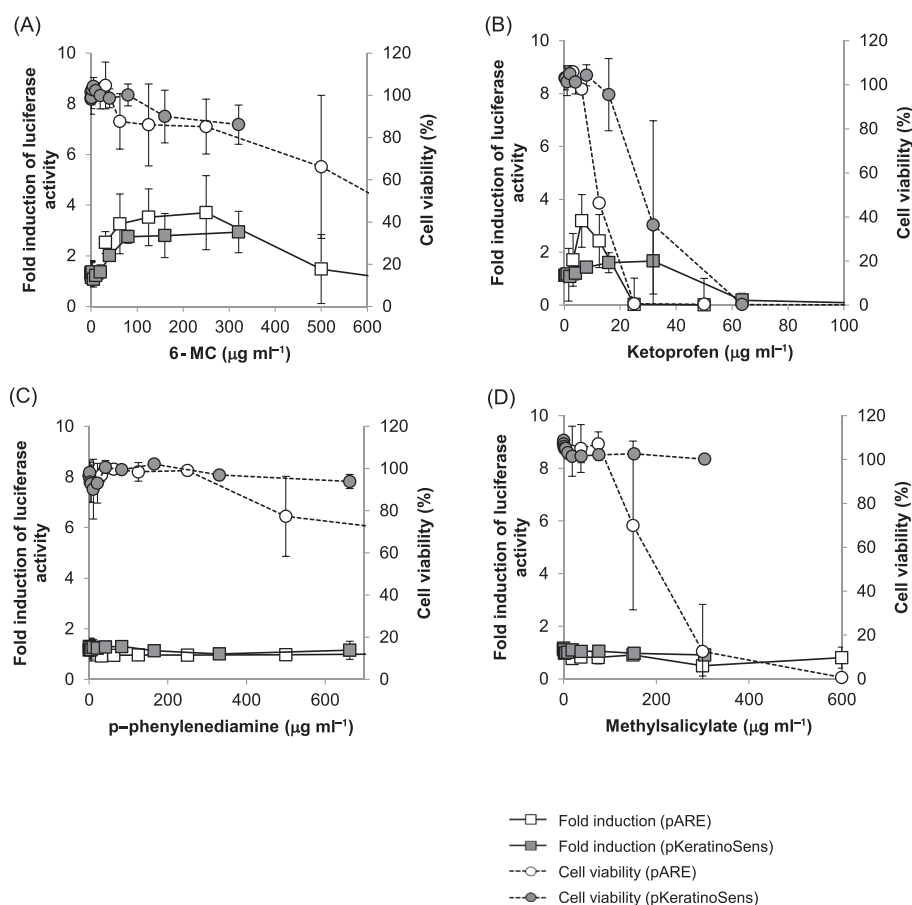


Figure 8. Comparison between cellular responses in photo-ARE and photo-KeratinSensTM for (A) 6-MC, (B) ketoprofen, (C) p-phenylenediamine and (D) methylsalicylate. Cells were irradiated with 5 J cm⁻² UV after treatment with the test chemical. Induction (fold) of luciferase activity and cell viability were calculated. The results are expressed as the mean $\pm$ SD of at least three independent experiments.

Table 3. Chemical information and results of photo-KerationSens™ of nine chemicals.

No	Test chemicals	CAS No.	in vivo or clinical data		Judgement	N ^a	Results of photo ARE assay				Reference
			photoallergic	phototoxic			Imax ^b (UV+/UV-)	Cmax ^c (μM)	Average of fold induction at Cmax ^d (UV+)	Average of fold induction at Cmax ^d (UV-)	
Photoallergenic/phototoxic chemicals											
	6-Methylcoumarin	92-48-8	+	+	P	3/3	2.94	2000	3.82	1.28	Lovell and Jones (2000)
	Ketoprofen	22071-15-4	+	-	P	3/3	1.61	62.5	2.20	1.47	Lovell and Jones (2000)
	Piroxicam	36322-90-4	+	+	N	1/3	1.30	1.95	1.17	0.90	Hariya <i>et al.</i> (1993)
	p- Phenylenediamine	106-50-3	+	+	P	3/3	2.55	2000	9.06	3.38	LeVine (1984), National Toxicology Program (1999)
	Trichlorocharban	101-20-2	+	+	N	0/3	1.28	0.977	2.50	2.03	Onoue <i>et al.</i> (2013b)
	Sulfanilamide	63-74-1	+	+	N	0/3	1.24	0.977	1.11	0.90	Onoue <i>et al.</i> (2013b)
	5- Methoxypsoralen	484-20-8	-	+	P	3/3	25.63	0.977	34.21	1.33	Lovell and Jones (2000)
Non-phototoxic chemicals											
	Sodium lauryl sulfate	151-21-3	-	-	N	1/3	1.22	7.813	1.32	1.10	Lovell and Jones (2000)
	Methylsalicylate	119-36-8	-	-	N	0/3	1.17	1.953	1.34	1.15	Onoue <i>et al.</i> (2013b)
CAS No.; Chemical Abstract Service number. P; Positive, N; Negative.											
^a N: Number of repetitions with significant luciferase induction/number of repetitions made.											
^b Imax: The highest average value of fold induction of luciferase activity at each test concentration among the three repetitions.											
^c Cmax: Concentration for Imax.											
^d Average of fold induction at Cmax: The average value excluding data rejected because of cytotoxicity.											

CAS No.: Chemical Abstract Service number. P; Positive, N; Negative.

^aN: Number of repetitions with significant luciferase induction/number of repetitions made.^bImax: The highest average value of fold induction of luciferase activity at each test concentration among the three repetitions.^cCmax: Concentration for Imax.^dAverage of fold induction at Cmax: The average value excluding data rejected because of cytotoxicity.

Table 4. Correlation between *in vivo* or clinical data for (A) photoallergenic/phototoxic chemicals and (B) photoallergens and photo-KeratinSens™ results.

		Photo KeratinoSens™	
		Positive	Negative
(A) <i>in vivo</i> or clinical data	Photoallergenic/phototoxic chemicals	4	3
	Non-phototoxic chemicals	0	2
	Accuracy	67%	
	Sensitivity	57%	
	Specificity	100%	
(B) <i>in vivo</i> or clinical data	Photoallergenic chemicals	3	3
	Non-photoallergenic chemicals	1	2
	Accuracy	56%	
	Sensitivity	50%	
	Specificity	67%	

Grey shading indicates that the same results were obtained in *in vivo* or clinical data and in photo-KeratinSens™

be avoided by conducting irradiation in PBS. Appropriate irradiation conditions for the test were 5 J cm^{-2} at the intensity of 1.7 m cm^{-2} , as determined with 6-MC as a representative photoallergen. The conditions are identical to those of the 3T3 NRU PT (OECD, 2004). The UV dose of 5 J cm^{-2} was reported to be sufficient for eliciting photocontact allergic test reactions (Hasan and Jansen, 1996).

Next, we evaluated various known photoallergens and/or phototoxins. Comparison with a non-irradiated sample is essential for distinguishing photoallergens, because the ARE assay was originally reported as an *in vitro* skin sensitization assay. The results indicated that 50% induction of luciferase expression compared with the vehicle control and the non-irradiated control would be a reasonable cut-off value for detecting photoallergens.

With this criterion, the accuracy of the photo-ARE assay as compared with *in vivo* or clinical data was 70%. Among the false negatives, sulfanilamide has absorption in the UVB region. It is difficult to detect photoallergens that absorb in the UVB region with our protocols because we used an H1 filter to attenuate UVB, which exhibits strong cytotoxicity. Trichlorocarbon caused significant luciferase induction in both irradiated and non-irradiated conditions, but the ratio was less than the positivity criterion of 1.5. Its potential as a skin sensitizer might be greater than that as a photoallergen. Indeed, there has been a case report of a positive reaction in a human patch test (Agren-Jonsson and Magnusson, 1976). Benzidamine HCl and tenoxicam exhibited luciferase induction in response to UV irradiation, but the ratio of irradiated to non-irradiated values did not exceed 1.5 so that these were false-negatives. Piroxicam caused no luciferase induction regardless of UV irradiation. As the Keap1–Nrf2–ARE pathway is activated by chemicals that bind to thiols of Keap1, piroxicam might bind instead to amines.

Although the accuracy of the photo-ARE assay versus *in vivo* data was slightly increased for photoallergenic chemicals (77%) as compared with photoallergenic/phototoxic chemicals (70%), specificity was decreased from 100% for photoallergenic/phototoxic chemicals to 85%. Photoallergenic chemicals may vary considerably in phototoxic potential (Kurita *et al.*, 2007), and there is no gold-standard method for evaluating photoallergenicity. In addition, phototoxic and photoallergic reactions often have a similar clinical and

histologic appearance (Gould *et al.*, 1995), and it is difficult to distinguish clearly the two. Hence, we consider this *in vitro* assay to be a photosafety test, rather than a photoallergenicity test. The key issues are the lack of an adequate definition of photoallergenicity and the absence of a reliable *in vivo* database of photoallergenic chemicals identified using a unified test method. Because of this difficulty of distinguishing photoallergenic and phototoxic chemicals, the present proposal must be considered tentative. The ARE assay and KeratinoSens™ were reported as *in vitro* skin sensitization assays and, therefore, these two assays reported here are supposed to detect photoallergens from the consideration of the photoallergenic mechanism.

In the case of photo-KeratinoSens™, luciferase induction was not observed with 6-MC, in contrast to the photo-ARE assay. In the original protocol of the ARE assay, cells were seeded at 50 000 cells per well and test chemicals were applied 48 h later, whereas in the original protocol of KeratinoSens™, cells were seeded at 10 000 cells per well and test chemicals were applied 24 h later. We considered one of the reasons why luciferase expression was not enhanced in response to UV irradiation in photo-KeratinoSens™ might be that the number of cells was insufficient, and so we examined the cellular response with increased numbers of cells. When cells were seeded at 30 000 cells per well, luciferase induction was observed in the presence of 6-MC with UV irradiation, so we decided to change the cell number per well from that of the original protocol. Appropriate irradiation conditions (5 J cm^{-2} at the intensity of 2.5 mW cm^{-2}) were also set using 6-MC as a model.

It should be noted that some compounds showed differences in cytotoxicity in photo-ARE and photo-KeratinoSens™, and this may be due in part to the difference in cell lines used in the two assays, as well as the difference in the actual UV dose (the dose in photo-KeratinoSens™ was about 60% of that in photo-ARE assay).

Comparing the results of the photo-ARE assay and photo-KeratinoSens™ for nine chemicals, positive or negative judgments were almost the same except for 5-methoxypsoralen, which was negative in the photo-ARE assay but positive in photo-KeratinoSens™ (Table 3). The accuracy of the photo-ARE assay and photo-KeratinoSens™ was almost the same for photoallergenic/phototoxic chemicals, but this should be

confirmed with a larger number of chemicals. Nevertheless, our results indicate that the photo-ARE assay and photo-KeratinoSensTM are useful as screening methods to predict photoallergenic and phototoxic potentials of chemicals. In contrast to skin sensitization, there is no standardized *in vivo* method for evaluating photoallergenicity, in terms of either category classification or threshold concentration. Therefore, it is difficult to predict the photoallergenic potency. However, it might at least be possible to identify potentially hazardous chemicals by combining multiple *in vitro* tests/*in silico* methods for the evaluation of skin sensitization (Natsch *et al.*, 2009; Jaworska *et al.*, 2011; Nukada *et al.*, 2013; Tsujita-Inoue *et al.*, 2014, 2015).

We previously reported an *in vitro* photoallergy assay that detects changes in CD86 and CD54 expression on human monocyte-derived cells (Hoya *et al.*, 2009) by adapting h-CLAT, which is now under consideration as an OECD guideline, for photoallergy testing. Furthermore, we are developing an *in vitro* method focused on antigen production for detecting photoallergens (unpublished data). Another test focused on antigen formation, photobinding of chemicals to human serum albumin, has also been used to evaluate the photoallergenic potential (Lovell and Jones, 2000). In addition, the ROS assay, which focuses on the energy transition of chemicals exposed to UV irradiation, has been developed as a photosafety test and adopted as an ICH S10 guideline. Thus, several *in vitro* methods, including the methods described here, that focus on different mechanistic aspects of photoallergenicity and/or phototoxicity are available. We believe that the use of appropriate combinations of these tests will make it possible to predict effectively the photoallergenic and phototoxic potential shortly.

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Conflict of interest

The Authors did not report any conflict of interest.

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