Research Article

Comparison of *in chemico* Skin Sensitization Methods and Development of *in chemico* Skin Photosensitization Assays

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Abstract

Chemical substances that induce an allergic response in skin upon contact are called skin allergens or sensitizers, while chemical substances that elicit an allergic response only in presence of light are called photoallergens or photo sensitizers. The Direct Peptide Reactivity Assay (DPRA, OECD Nº 442C, 2015) and the Amino Acid Derivative Reactivity Assay (ADRA) are *in chemico* assays used to discriminate between allergens and non-allergens. The DPRA and the ADRA, respectively, monitor the depletion of model peptides and modified amino acids induced by crosslinking with test chemicals. In the current study we compared these two assays and analyzed their suitability to predict skin sensitization potential of several chemical substances. In order to study the combined effect of a chemical compound and UV light, we modified DPRA (photo-DPRA) as well as ADRA (photo-ADRA) by introduction of a photo-irradiation parameter. Analysis using photo-DPRA and photo-ADRA correctly distinguished known photoallergens from non-photoallergens. Upon irradiation, photoallergens selectively showed higher depletion of model peptides or modified amino acids. Thus, photo-DPRA and/or photo-ADRA can serve as non-animal *in vitro* methods for the identification and assessment of photoallergens/photosensitizers.

1 Introduction

Allergic contact dermatitis (ACD) induced by repeated skin exposure of low molecular weight chemicals is characterized by skin rash, itch, and other complications such as erythema and edema. Chronic exposure may lead to skin thickening and eczema with cracks from itching. Exposure to allergens causes T-cell activation and proliferation that triggers adverse effects resulting in the dermal symptoms of ACD (Silvestre et al., 2018). Skin allergens can be identified using epidemiological data, clinical case studies and sensitization/ allergy tests (patch or maximization test) in humans (Basketter and Safford, 2016). Traditionally, animal based *in vivo* methods such as the mouse Local Lymph Node Assay (LLNA) and the guinea pig maximization test were used for the same purpose (Kimber et al., 2001). However, regulatory requirements borne out of ethical considerations are driving the development of non-animal alternative tests.

Regulatory bodies across the globe, including, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and Food and Drug Administration (FDA, US), recommend multiple Integrated Testing Strategies (ITS) using non-animal test batteries that combine *in silico*, *in chemico*, and *in vitro* methods to identify skin allergens (EURL ECVAM, 2013; Strickland et al., 2017; Daniel et al., 2018; Roberts and Patlewicz, 2018; Prior et al., 2019). ITS suggested for the skin allergy predictions proposes an assessment of test chemicals using multiple OECD adopted non-animal *in vitro* methods such as the Direct Peptide Reactivity Assay (DPRA) (OECD, 2015), KeratinoSensTM (OECD, 2018a), and human Cell Line Activation Test (h-CLAT) (OECD, 2018b) (Hoffmann et al., 2018). These three test methods are based on the Adverse Outcome Pathway (AOP), described by OECD, which defines the molecular initiating event (MIE) as well as key events (KEs) that link the sequence of causal incidences with manifestation of adverse health or environmental effects (Sewell et al., 2018). The skin allergenic response begins with the formation of covalent adducts between the chemical allergen and endogenous proteins that constitutes the MIE, or KE1, followed by a keratinocyte inflammatory response (KE2), activation of dendritic cells (KE3) and T-cell proliferation in lymph nodes (KE4) (OECD, 2014; Schultz et al., 2016).

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The DPRA (OECD N° 442C) (OECD, 2015) and ADRA (Fujita et al., 2014; Gerberick, 2016) are *in chemico* assays that are designed to investigate the MIE and distinguish between allergens and non-allergens by measuring the extent of covalent crosslinking of test chemicals with model peptides or modified amino acids, respectively. The DPRA is a simple, versatile, and widely used assay that evaluates reactivity of a test chemical with synthetic heptapeptides containing either cysteine (Cys-peptide) or lysine (Lys-peptide), whereas ADRA evaluates the crosslinking of test chemicals with novel cysteine derivative, N-(2-(1-naphthyl) acetyl)-1-cysteine (NAC) and novel lysine derivative, α -N-(2-(1-naphthyl) acetyl)-1-lysine (NAL). Both, DPRA and ADRA, analyze peptide reactivity indirectly by determining percent (%) depletion of model heptapeptides, and NAC and NAL, respectively. For the DPRA, a threshold of 6.38% average for Cys- and Lys-peptide depletion is used to discriminate between allergens and non-allergens, while for Cys-peptide alone this threshold is 13.89% (OECD, 2015). The suggested average % NAC and NAL depletion threshold value for ADRA is 7.750%. Thus, model peptides or NAC and NAL depletion data can be used to study the skin allergy hazard classification and to distinguish between allergens and non-allergens of a test substance (Fujita et al., 2014). The data presented herein, validate the utility of DPRA in predicting the nature of low molecular weight chemical compounds, as per OECD 442C guidelines. The ADRA prediction closely matches with those of DPRA suggesting its usefulness.

Most chemical allergens contain an electrophilic group that allows direct covalent binding with skin proteins to form immunogenic protein-hapten adducts. A significant number of sensitizers without electrophilic moieties require biotic (mediated by interaction with skin enzymes) or abiotic (mediated by interaction with air/ water oxygen, radiation) transformation and are accordingly named as pro-haptens and pre-haptens, respectively (Urbisch et al., 2016). Chemical substances and drugs, such as tetracycline, piroxicam, chlorpromazine (CPZ), amiodarone, and naproxen are pre-haptens that do not show intrinsic peptide reactivity and act as sensitizers only in combination with light (Drucker and Rosen, 2011). Topical and systemic exposure of such drugs, cosmetics or other environmental chemicals in presence of UV light can induce cutaneous manifestations such as sun burn dermatitis, planus lichenoids, etc. (Sharma et al., 2013), which can be phototoxic and/or photoallergic (photosensitizing) in nature (Glatz and Hofbauer, 2012). Phototoxic reactions induce direct tissue damage due to reactive oxygen species, whereas photoallergic reactions induced by photosensitizers / photoallergens resemble ACD. We present here data from modified DPRA (photo-DPRA) and ADRA (photo-ADRA), which supports the successful application of these *in chemico* assays in prediction of photoallergy induced by test chemicals.

2 Materials and Methods

Chemicals and reagents

Hepta-peptides containing Cysteine (Ac-RFAACAA-COOH) or Lysine (Ac-RFAAKAA-COOH) were obtained from RS Synthesis, Louisville, KY 40270, USA. Purity of both peptides was above 95%. NAC and NAL were custom synthesized and purchased from GRK Research laboratories, private limited, Hyderabad, India. Purity of NAC and NAL was confirmed using 1H NMR (data not shown). Table 1 contains the list of chemicals used in the study and their suppliers. All other chemicals and solvents were obtained from Sigma.

DPRA

DPRA was performed as per OECD 442C guidelines. Briefly, Cysteine and Lysine peptide stock solutions were prepared at 0.667 mM final concentration, in 100 mM phosphate buffer (pH 7.5) and ammonium acetate buffer (pH 10.2), respectively. 100 mM stock solutions of test chemicals were prepared in acetonitrile or in the indicated solvent (Tab.1). All samples were prepared in triplicates. 750 µl Cys-peptide was mixed with 200 µL acetonitrile, and 50 µL test chemical representing 0.5 mM of the peptide and 5 mM of the test chemical in 1:10 mM ratio. The Lys-peptide reaction contained 750 µL Lys-peptide from stock solution, 250 µL of test chemical, providing 0.5 mM of the peptide and 25 mM of the test chemical in 1:50 mM ratio. Peptides at 0.0167, 0.0334, 0.0668, 0.1335, 0.2670 and 0.534 mM were used to generate a standard calibration curve. Reaction vials were incubated in the dark at 25 °C for $24 \pm$ 2 h. Next, samples were analyzed by reverse-phase HPLC (Agilent technology 1100 series: Chemstation, Shimadzu LC2010CHT: LC solutions) with UV detection at wavelength 220 nm using Zorbax SB-C-18 2.1 mm x 100 mm x 3.5-micron column and C18 guard cartridge (2.0×4.0 nm). The system was equilibrated at 30°C with 50% mobile phase A (0.1% (v/v) trifluoroacetic acid in water) and 50% phase B (0.085% (v/v) trifluoroacetic acid in acetonitrile) for at least 2 h before running samples. 5 µL samples were injected and separation was achieved using a 0.35 mL/min flow rate with a gradient of mobile phase over a 20 min period. The gradient was adjusted from 90% A/ 10% B to 10% A /90% B during the initial 13 min. The gradient was then rapidly returned to 90% A/10% B over 30 min. The concentration of peptide was determined in each reaction from absorbance at 220 nm. The appropriate peak was integrated yielding the peak area corresponding to the individual unreacted peptide in the test reactions, controls, and standards. A linear calibration curve was prepared based on the peptide concentration standards. The calibration curve was generated by plotting peak area vs peptide concentration. The calibration curve was considered valid if the correlation coefficient (r^2) was > 0.990.

ADRA

ADRA was carried out according to methods described by Fujita and co-workers (Fujita et al., 2014). Briefly, 1.25 mM NAC was dissolved in 100 mM phosphate buffer (pH 9.5) and 1.25 mM NAL was dissolved in 100 mM phosphate buffer (pH 12.0). Test chemical stock solutions were prepared in acetonitrile/water at 100 mM. Stock solutions of amino acid derivatives, test chemicals, acetonitrile and phosphate buffer, were mixed, and 80 μ L of NAC/NAL stock solution, 70 μ L of Buffer, 40 μ L of acetonitrile and 10

 μ L (for NAC) or 50 μ L (for NAL) of test chemicals were mixed and incubated in the dark for 24 ± 2 h at 25°C. Post incubation, samples were diluted 10 times with a solution of 25% acetonitrile and 0.5% trifluoroacetic acid (TFA) in water. 10 μ L of NAC and NAL samples were injected and their levels were quantified using HPLC-UV (HPLC-Agilent technology 1100 series, Shimadzu LC2010CHT) at 281 nm wavelength. Zorbax SB-C-18 2.1 mm x 100 mm x 3.5 micron column and C18 guard cartridge (2.0 ×4.0 nm) were used.

Sr. No.	Test chemicals	Source	CAS N°	Soluble in
1	DNCB	Sigma	97-00-7	Acetonitrile
2	Oxazolone	Sigma	15646-46-5	Acetonitrile
3	Formaldehyde	Sigma	50-00-0	Acetonitrile
4	Benzylideneacetone	Sigma	122-57-6	Acetonitrile
5	Farnesal	Sigma	19317-11-4	Acetonitrile
6	2,3-Butanedione	Sigma	431-03-8	Acetonitrile
7	1-Butanol	Sigma	71-36-3	Acetonitrile
8	6-Methylcoumarin	Sigma	92-48-8	Acetonitrile
9	Lactic Acid	Flurochem Ltd.	50-21-5	Acetonitrile
10	4-Methoxyacetophenone	Sigma	100-06-1	Acetonitrile
12	Cinnamyl Alcohol	Sigma	104-54-1	Acetonitrile
13	EGDMA	Sigma	150-38-9	Acetonitrile
14	2-Mercaptobenzothiazole	Sigma	149-30-4	Acetonitrile
15	4-Methoxyamino Hemisulfate	Sigma	55-55-0	Water
16	2-Propanol	Sigma	67-63-0	Acetonitrile
17	Salicylic Acid	Sigma	69-72-7	Acetonitrile
18	Glycerol	Sigma	56-81-5	Water
19	Chloramine-T	Sigma	7080-50-4	Water
20	Phenyl acetaldehyde	Sigma	122-78-1	Acetonitrile
21	Vanillin	Sigma	121-33-5	Acetonitrile
22	Imidazolidinyl Urea	Sigma	39236-46-9	Water
23	Guaicol	Sigma	90-05-1	Acetonitrile
24	Phenol	Sigma	108-95-2	Acetonitrile
25	Ethanolamine	Sigma	141-43-5	Acetonitrile
26	Furaltadone	Sigma	139-91-3	Acetonitrile
27	Propylene Glycol	Sigma	57-55-6	Acetonitrile
28	Glutaraldehyde	Sigma	111-30-8	Acetonitrile
29	Amiodarone HCL	Sigma	19774-82.4	Acetonitrile:Water
30	Chlorpromazine HCL	Sigma	69-09-0	Acetonitrile
31	Anthracene	Sigma	120-12-7	Acetone
32	Protoporphyrin IX, Disodium	Sigma	50865-01-5	Acetonitrile
33	L-Histidine	Sigma	5934-29-2	Water
34	Hexachlorophene	MP Bio medicals	70-30-4	DMSO
35	Sodium lauryl sulfate	Sigma	151-21-3	Water
36	Norfloxacin	Sigma	70458-96-7	Ammonium acetate buffer, pH 10.2

 Tab 1: Commercial sources, CAS N°, and solvents used for dissolving the test chemicals

 Abbreviations, EGDMA: Ethylene glycol dimethacrylate, DNCB: 2,4-dinitrochlorobenzene

The mobile phase had a flow rate of 0.3 mL/min, it consisted of A (98:02 mixture of water: acetonitrile with 0.1% trifluoroacetic acid) and B (90:10 mixture of acetonitrile: water with 0.1% trifluoroacetic acid). Separation was achieved by holding at the initial condition (20% B) for 7 min followed by a linear gradient to 100% B for 3.5 min and then back to the initial condition for a total analysis time of 20 min per sample. Mobile phase conditions were altered for test chemicals that co-eluted with NAL: phenylacetaldehyde and guicacol (gradient starting linear at 20% B for 2 min to 50% B till 12 min, to 100% B till 12.5 min, linear 100% B till 15 min, linear 35% B till 20 min, flow rate 0.3 mL/min), and oxazolone and 2-mercaptobenzothiazole (gradient starting at 45% B to 55% B for 10 min, followed by 100% B for 10.5 min to 13.5 min, 45% B linear from 13.5 min to 20 min, flow rate 0.2 mL/min, run time 20 min). Mobile phase conditions were altered for 6-methylcoumarin, which co-eluted with NAC (gradient starting at 35% B linear for 2 min, followed by 35% B to 60% B till 12 min, to 100% B till 12.5 min, linear 35% B till 20 min, followed by 35% B to 60% B till 20 min, followed by 35% B to 60% B till 20 min, to 100% B till 12.5 min, linear 100% B till 15 min, linear 35% B till 20 min, followed by 35% B to 60% B till 20 min, to 100% B till 12.5 min, linear 100% B till 15 min, linear 35% B till 20 min, followed by 35% B to 60% B till 20 min, to 100% B till 12.5 min, linear 100% B till 15 min, linear 35% B till 20 min, followed by 35% B to 60% B till 20 min, to 100% B till 12.5 min, linear 100% B till 15 min, linear 35% B till 20 min, flow rate 0.3 mL/min).

Photo-DPRA and photo-ADRA

All chemicals were made at 100 mM stock, except anthracene and norfloxacin. These were prepared at 50 mM and 33.33 mM, respectively, due to their lower solubility. Accordingly, the final concentration of these compounds in the assay is lower compared to other chemicals. Most parameters used for DPRA and ADRA were kept unchanged for conducting photo-DPRA and photo-ADRA. Test chemicals were mixed with model peptides or NAC/NAL and the mixture was exposed to UVA to get 5 J/cm² dose. Intensity of light (irradiance) was checked before each experiment using a broadband UV meter and exposure time was adjusted to achieve the dose of 5 J/cm². Reaction vials were incubated in the dark at 25°C for 24 ± 2 h and HPLC analysis was performed, as described in DPRA and ADRA methods. The UV cabinet was sourced from Scientific Sales Syndicate (N P Botny model); it was equipped with UVA (365 nm), UVB (254 nm) and visible light lamps. A handheld digital UV meter from Sai Water Equipment was used to measure the light intensity. It utilizes a stable photovoltaic sensor with a UV pass filter that blocks visible and infrared radiation.

Statistics

Each experiment was repeated at least three times. Mean % depletion values of heptapeptides and modified amino acids from three or more independent experiments are shown in the results section. Standard deviation (SD) and Relative Coefficient of Variability (RCV) values were calculated and are indicated in the respective tables. SD and RCV values are useful in determining the data accuracy and reproducibility. Generation of DPRA and ADRA calibration curves is described in supplementary data S2 and S6¹, respectively. As per the DPRA acceptance criteria (described briefly below) mentioned in the OECD guidelines, interpretation of the results must be based on the r^2 (square of the coefficient of correlation) value obtained from standard calibration curves of Cys- and Lys-peptides as well as SD and RCV values of reference controls, positive control and test chemicals. If these criteria were not met, the data were rejected and the run was repeated. Note that the r^2 value close to unity indicates the linear correlation between UV absorbance and varying concentrations of Cys / Lys-peptide and NAC / NAL, in DPRA and ADRA, respectively.

DPRA acceptance criteria

The following criteria should be met for a run to be considered valid: a) The standard calibration curve should have an $r^2 > 0.99$. b) The mean percent peptide depletion value of the three replicates for the positive control cinnamaldehyde should be between 60.8% and 100% for the cysteine peptide and between 40.2% and 69.0% for the lysine peptide and the maximum SD for the positive control replicates should be <14.9% for the percent cysteine depletion and <11.6% for the percent lysine depletion. c) The mean peptide concentration of reference control A should be 0.50 \pm 0.05 mM and the RCV of peptide peak areas for the nine reference controls B and C in acetonitrile should be <15.0%.

The following criteria should be met for a test chemical's results to be considered valid: a) The maximum SD for the test chemical replicates should be <14.9% for the percent cysteine depletion and 11.6% for the percent lysine depletion. b) The mean peptide concentration of three reference controls C in the appropriate solvent should be 0.50 ± 0.05 mM. Some batches of acetonitrile have a negative impact on Cys-peptide stability. As per guidelines, acetonitrile batches that show <15% RCV in the Cys-peptide stability should be used.

3 Results

3.1 Fulfillment of DPRA acceptance criteria as per OECD guidelines

Synthetic Cys- and Lys-peptides of purities 98.2 % and 98.4%, respectively, were obtained and stock solutions of 0.667 mM were made for both peptides and their detection as well as separation method were established using HPLC-UV, as described in OECD 442C (OECD, 2015). As recommended by EURL ECVAM guidelines, we assessed an influence of the mobile phase component acetonitrile on the stability of the Cys-peptide (Tab. S1A¹) at 25 °C for 48 h (EURL ECVAM, 2013). Additionally, the stability of the Lys-peptide (Tab. S1B¹) in acetonitrile for 24 h was also checked. The concentration of Cys-peptide at 0 h, 24 h, 48 h and of Lyspeptide at 0 h and 24 h were determined by using calibration curve data (Fig. S2B, S2C; Tab. S2D¹). Note that the concentration of both Cys- and Lys-peptide standards were prepared as described (Tab. S2A¹) and calibration curves for both peptides were generated using concentrations from 0.534 mM to 0.0167 mM (Fig. S2B, S2C¹). Both peptides showed a linear correlation between dose and UV

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absorption at 220 nm ($r^2 \ge 0.99$). r^2 values for four different calibration experiments are shown in Tab. S2D¹. $r^2 \ge 0.99$ fulfils the first OECD acceptance criteria. Next, as per guidelines, we also met the second major acceptance criteria for the positive control cinnamaldehyde (Fig. 1). Figure 1 shows the representative chromatograms of reference control B obtained from the Cys-peptide (Fig. 1A) and Lys-peptide (Fig. 1B) in acetonitrile solvent after 24 h incubation, as well as of the samples containing Cys-peptide (Fig. 1C) and Lys-peptide (Fig. 1D) after 24 h incubation with cinnamaldehyde. Mean % depletion values for Cys- and Lys-peptides were determined by comparing AUC (area under curve) of sample (cinnamaldehyde) and of reference control B according to Formula 1:

Percent Pentide Depletion= $\begin{bmatrix} 1-t \end{bmatrix}$	Peptide peak area in replicate injection	
	Mean peptide area in reference controls C/J ^{A roc}	

Mean % peptide depletion values of four replicates and average (Cys + Lys) % peptide depletion are presented in Fig. 1E. Note that the % peptide depletion values are within the expected ranges of 60.8 to 100 for Cys-peptide, and 40.2 to 69 for Lys peptide. The RCV for replicates of Reference Control B that contain only peptides with acetonitrile (solvent) were 0.76, 1.04, 0.89, 3.21 for the Cys-peptide and 0.37, 1.39, 0.94 and 1.67 for the Lys-peptide. For the positive control cinnamaldehyde, RCV values of the % depletion for Cys- and Lys-peptides were < 14.9 and < 11.6, respectively (Fig. 1E). These values are within acceptable ranges as described in OECD 442C.



Fig. 1: % peptide depletion for DPRA positive control

A-D) Levels of Cys-peptide in 0.667 mM, pH 7.5 phosphate buffer (A) or Lys-peptide in 0.667 mM, pH 10.2 ammonium acetate buffer (C) (Reference Control B) and Cys-peptide with cinnamaldehyde (B) or Lys-peptide with cinnamaldehyde (D) were analyzed by HPLC-UV, after incubation at 25 ± 2.5 °C for 24 ± 2 h. Retention time for Cys-peptide is 9.4 min and for Lys-peptide it was 6.7 min, as indicated by arrows and observed peaks in the representative chromatograms. E) Individual % depletion values for Cys- and Lys-peptide for four independent replicates and average (Avg) % depletion as well as standard deviation (SD) were calculated and are indicated in the table.

3.2 Validation of DPRA assay using OECD 442C proficiency chemicals

Next, we performed the DPRA for 10 proficiency compounds described in the OECD 442C guidelines (Tab. 2). Individual compounds were incubated with either Cys- or Lys-peptide for 24 h at 25°C, followed by their detection using HPLC-UV after 24 h incubation period. Retention times for Cys- and Lys-peptides were 9.4 min and 6.7 min, respectively. A co-elution control was used to check overlapping elution of peptide with compound, if any. Ratio of the peptide to test chemical was maintained at 1/10 for Cys- and 1/50 for Lys-peptide. Reference controls that contains peptide with solvent (acetonitrile) at 0 h (reference control A) and after 24 h incubation (reference control B) were made at the same time, followed by immediate analysis of reference control A, whereas reference control B samples were analyzed after indicated incubation periods at 25 ± 2.5 °C (Tab. S3¹). Analysis of the co-elution control containing only test chemicals revealed that none of the 10 proficiency chemicals co-eluted with model peptides, as indicated by distinct retention times (Tab. S4¹).

% depletion values for Cys- and Lys- peptides were determined by comparing AUC of sample and of reference control B according to Formula 1. Respective values with corresponding SD for each compound are shown in Figure 1E. Note that SD values are below 14.9% and 11.6% for Cys- and Lys-peptides, respectively, for all tested compounds, indicating fulfilment of acceptance criteria. Observed peptide depletion values for each compound are within the expected ranges, except for % Cys-peptide depletion value of 66.25 of formaldehyde which is higher than the expected range of 30 - 60. Average % depletion values for both peptides were calculated and used to classify test substances, according to the prediction model in Table S5¹. In accordance with the OECD 442C test guideline, out of 10 proficiency substances, six sensitizers were predicted correctly with average % depletion values in the range of 18% - 66%, whereas % depletion values for 4 compounds below the 6.38% cut off also correctly predicted them to be non-sensitizers.

Tab 2: Analysis of DPRA proficiency chemicals

Ten proficiency chemicals described in OECD 442C guidelines were incubated with model Cys- or Lys-peptides at 25 ± 2.5 °C and levels of each peptide after 24 ± 2 h incubation were analysed by HPLC-UV. % depletion of each peptide caused by respective chemical was calculated using formula 1. Values represent mean of four independent experiments with three replicates in each experiment. Avg % peptide depletion (Cys + Lys) values were used to classify test substances according to the prediction model in Fig. S5. Note that predictions matched with expected results, as described by OECD 442C guidelines. Actual here indicates observed depletion values whereas Exp represents expected range of % depletion values as per 442C guidelines. (Abbreviations, SD: standard deviation, S: sensitizer, NS: non-sensitizer, DNCB: 2,4-dinitrochlorobenzene).

Sr.	Test Chemisel	% Cys- peptide Depletion			% Lys-peptide Depletion			Avg % D	epletion	Prediction	
No.	Test Chemical	Actual	SD	Ехр	Actual	SD	Exp	Actual	SD	Actual	Exp
1	DNCB	100.00	0.00	90-100	30.02	1.01	15-45	65.01	0.51	S	S
2	Oxazolone	73.69	1.13	60-80	52.03	0.84	10-55	62.86	0.34	S	S
3	Formaldehyde	66.23	0.54	30-60	7.07	0.69	0-24	36.65	0.36	S	S
4	Benzylideneacetone	93.04	0.08	80-100	1.20	0.23	0-7	47.12	0.14	S	S
5	Farnesal	31.50	1.15	15-55	5.30	0.56	0-25	18.40	0.71	S	S
6	2-3 Butanedione	64.12	0.58	60-100	20.33	1.79	10-45	42.23	1.10	S	S
7	1-Butanol	1.99	0.38	0-7	0.50	0.15	0-5.5	1.24	0.23	NS	NS
8	6-Methylcoumarin	2.11	0.50	0-7	0.00	0.54	0-5.5	1.05	0.25	NS	NS
9	Lactic acid	1.73	1.56	0-7	0.13	0.59	0-5.5	0.94	0.82	NS	NS
10	4-Methoxy acetophenone	1.42	1.32	0-7	0.30	2.13	0-5.5	0.87	0.76	NS	NS

3.3 Determination of sensitization potential for 10 proficiency compounds using ADRA assay

ADRA was first described by Fujita and co-workers (Fujita et al., 2014). It makes use of novel Cysteine (NAC) and Lysine (NAL) derivatives containing a naphthalene ring attached to an α amino group. NAC and NAL were freshly prepared before each experiment and their separation as well as detection methods were established using HPLC-UV. The calibration curves for NAC and NAL showed linear correlations between the dose and UV absorption at 281 nm with $r^2 \ge 0.99$ (Fig. S6¹). Next, we performed ADRA with DPRA proficiency compounds using NAC and NAL. Cinnamaldehyde was included as a positive control (Fig. 2). Average % NAC and NAL depletion value was 64.30% which is close to the DPRA value. Reference control B (with solvent acetonitrile) and reference control C (with solvent water) concentrations at 24 h were calculated and used for determining % NAC and % NAL depletion during 24 h. Comparison of % NAC and NAL depletion values with the values reported by Fujita et al is shown in Table 3. In addition to the 10 proficiency chemicals (Tab. 3, No. 2-11), we also included 10 chemical substances described by Fujita et al. (Tab. 3, No. 12-21) and 5 other compounds (Tab. 3, No. 21-26). Reference controls, using respective solvents, were used for calculating % depletion. Elution controls were used to determine the retention times of test chemicals; altered mobile phase conditions for the chemicals that co-eluted with NAC or NAL are described in the methods section.



Fig. 2: % NAC and NAL depletion for cinnamaldehyde

A-D) 1.25 mM each of NAC and NAL in phosphate buffer of pH 9.5 and pH 12.5, respectively, were incubated with (B, D) or without cinnamaldehyde (A, C) for 24 ± 2 h at 25 ± 2.5 °C, followed by detection and analysis using HPLC-UV. Retention time for NAC is 3.3 min and for NAL is 2.8 min, as indicated by arrows in the representative chromatograms. E) Individual % depletion values for NAC and NAL peptide for three independent replicates (I-III) and average (Avg) % depletion as well as standard deviation (SD) were calculated and are shown in the table.

Individual % depletion values for certain chemicals such as oxazolone, farnesal, EGDMA and imidazolidinyl urea varied slightly from the expected value. Also, chemicals such as cinnamaldehyde, glutaraldehyde, formaldehyde, and vanillin, contain an aldehyde group which is particularly sensitive to changes in reaction conditions as it is likely to be oxidized to carboxylic acid. This may contribute to variations in reactivity of aldehydes and hence affect the % depletion values across the laboratories. Defining the upper and lower limit of % NAC and % NAL depletion values for individual chemicals by generating high quality reference data is essential to know, if these values are within acceptable ranges. This can be achieved by careful selection of commercially available ADRA proficiency substances to represent the range of skin sensitization hazard responses. High quality *in vivo* and *in vitro* reference data generated from EURL-ECVAM-coordinated validation studies has made it easier for the laboratories across globe to successfully implement DPRA. Similar approach that enables proficiency testing using reference chemicals will be essential to convincingly establish ADRA as an effective skin sensitization hazard examination tool.

Fujita et. al. have described a 2- class prediction model for ADRA depending on the average % NAC and % NAL depletion values. Comparison of average % depletion values showed that out of 21 chemicals that could be compared, the prediction of 20 matched in both studies (Tab. 4). As opposed to Fujita et al., vanillin was found to be a non-sensitizer in our studies (Fujita et al., 2014), however, their follow up studies with modified ADRA that employs 1/100 concentration of test chemical and shows higher sensitivity and accuracy, reported vanillin to be a non-sensitizer (Yamamoto et al., 2015). We also performed the DPRA assay for these 26 test chemicals and compared ADRA and DPRA results. Our ADRA and DPRA predictions matched for all tested chemicals (Tab. 4).

Tab 3: % NAC and NAL depletion in ADRA

Indicated chemicals were incubated with NAC or NAL for 24 ± 2 h at 25 ± 2.5 °C, followed by analysis of NAC and NAL levels using HPLC-UV. % NAC and NAL depletion values were calculated as described in methods. Values represent mean of three independent experiments with three replicates in each experiment. Obtained % NAC and NAL depletion values (Actual) were compared with average % NAC and NAL depletion reported by Fujita et. al. 2014. (Abbreviations, SD: standard deviation. N.A. not available).

- N			% NAC E	Depletion		% NAL Depletion				
Sr. No.	Test Chemical	Actual	SD	Fujita	SD	Actual	SD	Fujita	SD	
1	Cinnamaldehyde	87.97	0.85	95.5	1.6	40.62	7.93	81.30	1.80	
2	DNCB	100.00	0.00	100.0	0.0	77.68	3.29	83.20	0.90	
3	Oxazolone	63.61	0.51	76.5	0.4	0.00	1.09	30.10	1.80	
4	Farnesal	75.67	3.00	41.3	0.8	36.41	1.64	38.60	1.90	
5	Formaldehyde	83.90	0.96	81.7	1.9	50.24	2.77	65.60	2.40	
6	Benzylideneacetone	95.11	0.12	95.3	2.1	2.87	1.69	15.00	1.10	
7	2-3 Butanedione	100.00	0.00	100	0.0	72.62	3.49	73.10	2.80	
8	1-Butanol	0.00	1.32	3.9	2.4	2.48	0.37	2.50	1.60	
9	6-Methylcoumarin	2.93	0.93	6.1	1.8	1.42	1.34	4.50	1.00	
10	Lactic Acid	0.00	0.55	0.0	1.8	2.33	0.91	2.80	1.90	
11	4-Methoxyacetophenone	0.72	4.38	4.1	1.3	3.52	0.63	0.0	2.30	
12	EGDMA	97.97	0.14	100.0	0.0	4.10	0.91	24.30	1.30	
13	Glycerol	3.10	1.49	4.3	2.3	0.33	0.40	4.30	1.90	
14	Glutaraldehyde	100.00	0.00	49.2	0.6	42.89	1.83	96.20	1.40	
15	Imidazolidinyl Urea	66.69	1.14	80.0	0.5	73.15	1.18	83.70	1.00	
16	2-Mercaptobenzothiazole	48.70	2.48	40.0	1.9	0.0	1.41	0.0	0.80	
17	Phenylacetaldehyde	100.00	0.00	99.8	0.4	90.42	0.74	99.20	1.00	
18	Propylene glycol	0.00	1.56	0.0	2.1	0.0	0.69	0.0	1.00	
19	2-Propanol	0.00	0.64	0.0	1.8	0.0	1.78	0.0	6.70	
20	Salicylic Acid	0.00	1.65	0.0	1.4	11.36	4.41	1.50	0.70	
21	Vanillin	0.00	1.69	0.0	2.3	5.09	0.57	62.70	0.60	
22	Chloramine-T	100.00	0.00	N. A.	-	90.97	0.91	N. A.	-	
23	Cinnamyl alcohol	10.86	0.78	N. A.	-	1.56	0.77	N. A.	-	
24	Ethanolamine	8.80	3.94	N. A.	-	0.08	0.98	N. A.	-	
25	Furaltadone	85.45	1.84	N. A.	-	88.98	0.87	N. A.	-	
26	Guaiacol	8.73	3.43	N. A.	-	11.04	0.78	N. A.	-	

3.4 Modification of DPRA to evaluate photo-sensitization of test chemical

Next, in order to develop an *in chemico* assay that discriminates photo allergens from non-photo allergens, DPRA was modified to introduce additional step of a UV exposure. Test chemicals, described in the NRU 3T3 PT test guide, were used as reference compounds. The NRU 3T3 PT assay is an OECD (OECD, 2004) adopted method used to identify the phototoxic potential of test compounds. It relies on assessing the viability of 3T3 fibroblasts exposed to test chemical in the presence and absence of light. Phototoxic compounds such as CPZ, amiodarone, protoporphyrin IX, norfloxacin, and anthracene described in NRU 3T3 PT test guideline are also known to act as photoallergens (Drucker and Rosen, 2011). Prior to 24 h incubation, Lys- and Cys-heptapeptides were exposed to 5 J/cm² UVA in the presence of test chemical. Out of three UV radiations emitted by sun, UVA (320 - 400 nm), UVB (290 – 320 nm) and UVC (180 – 290 nm), UVA and UVB reach earth's surface, while UVC is blocked by ozone layer. Very few chromophores such as eosin, absorb light in the visible spectrum, whereas sunburn and high cytotoxicity even with small doses of UVB, limits its exposure tolerance (Palmer and White, 2006). Additionally, unlike UVA, UVB cannot penetrate the deeper dermis layer, and it is UVA, which is the more abundant radiation in the sunlight, moreover most photosensitizers also absorb UVA more efficiently than UVB. These facts support the observed higher contribution of UVA to phototoxic and photoallergic reactions

Tab 4: Comparison of skin sensitization predictions obtained from DPRA and ADRA analysis

Average Cys + Lys % peptide depletion and average NAC + NAL % depletion values obtained in DPRA and ADRA, respectively, for the indicated test chemicals are shown together with respective sensitization predictions. Additionally, average NAC + NAL % depletion values and respective sensitization predictions. reported by Fujita et. al. 2014 are shown for comparison of data. ADRA predictions were made as described by Fujita et. al., 2014, whereas DPRA predictions were based on OECD 442C guidelines. (Abbreviations, EGDMA: Ethylene glycol dimethacrylate, DNCB: 2, 4- dinitrochlorobenzene, S: sensitizer, NS: non-sensitizer, Pred: prediction).

Sr.	Test Chemical	ADRA (Fu	jita et. al.)	AD	RA	DPRA		
No.	Test Chemical	% Depletion	Pred	% Depletion	Pred	% Depletion	Pred	
1	Cinnamaldehyde	88.4	S	64.3	S	70.0	S	
2	DNCB	91.6	S	88.8	S	65.0	S	
3	Oxazolone	53.3	S	31.8	S	62.8	S	
4	Farnesal	39.9	S	56.0	S	18.4	S	
5	Formaldehyde	73.7	S	67.0	S	36.6	S	
6	Benzylideneacetone	55.1	S	48.9	S	47.1	S	
7	2-3 Butanedione	86.5	S	86.3	S	42.2	S	
8	1-Butanol	3.2	NS	1.2	NS	1.2	NS	
9	6-Methylcoumarin	5.3	NS	2.1	NS	1.0	NS	
10	Lactic Acid	0.2	NS	1.1	NS	0.9	NS	
11	4-Methoxyacetophenone	1.3	NS	2.1	NS	0.8	NS	
12	EGDMA	62.1	S	51.0	S	73.3	S	
13	Glutaraldehyde	72.7	S	71.4	S	40.4	S	
14	Glycerol	4.3	NS	1.7	NS	4.2	NS	
15	Imidazolidinyl urea	81.8	S	69.9	S	35.2	S	
16	2-Mercaptobenzothiazole	19.5	S	24.3	S	32.3	S	
17	Phenylacetaldehyde	99.5	S	95.2	S	59.7	S	
18	Propylene Glycol	0.00	NS	0.0	NS	2.9	NS	
19	2-Propanol	0.00	NS	0.0	NS	2.2	NS	
20	Salicylic Acid	0.00	NS	5.6	NS	6.8	NS	
21	Vanillin	31.3	S	2.5	NS	4.7	NS	
22	Chloramine-T	NA	S	95.4	S	100.0	S	
23	Cinnamyl alcohol	NA	NS	6.2	NS	5.5	NS	
24	Ethanolamine	NA	NS	4.4	NS	4.6	NS	
25	Furaltadone	NA	S	87.2	S	52.0	S	
26	Guaiacol	NA	S	9.8	S	16.9	S	

compared to UVB or visible light. Hence, to be closer with the actual phenomenon, our studies used UVA radiation which is more relevant, to investigate the skin photosensitization hazard of test chemicals. Reference and elution controls were made as described above. The standard curves for Cys- and Lys-peptides were generated as described earlier. Mean % depletion values of DPRA positive control cinnamaldehyde for Cys- and Lys-peptides were 82.93% and 65.86%, respectively, as expected.

Cys- and Lys-peptide % depletion values with and without UVA irradiation are shown in table 5. A strong increase in % peptide depletion upon UVA exposure was seen for CPZ, amiodarone HCL, anthracene and norfloxacin suggesting their potent photosensitizing nature. This increase in depletion was mostly observed in the Cys-peptide. A moderate increase in % peptide depletion was observed for protoporphyrin IX. Whereas, the known photosensitizer hexachlorophene which does not show phototoxicity in NRU 3T3 PT assay also showed subtle increase in % depletion upon UVA exposure (Miyachi and Takigawa, 1983). % peptide depletion values for sodium lauryl sulfate and L-histidine did not change significantly with or without UV exposure indicating their non-sensitizing nature in this assay. Note that the DPRA assay is not suited to study sensitization potential of strong

detergents like sodium lauryl sulfate (SDS) due to its ionic interactions with the model peptides. This is supported by selective depletion of cationic Lys-peptide in presence of negatively charged SDS (Tab. 5) and false positive detection of SDS as a sensitizer in DPRA.

Tab 5: Analysis of photo sensitization of NRU 3T3 PT proficiency chemicals by photo-DPRA

Indicated test chemicals were exposed to UV in presence of Cys- or Lys-heptapeptides. Exposure time was adjusted to achieve a dose of 5J/cm² depending upon the measured irradiance. Control non-exposed (- UV) and UV exposed (+ UV) samples were further incubated at 25 ± 2.5 °C for 24 ± 2 h and peptides were separated, measured by HPLC-UV. % depletion values of Cys- and Lys-peptide with or without UV exposure were calculated as described in methods. Values represent mean of three independent experiments with three replicates in each experiment. (Abbreviation, SD: standard deviation, SDS: Sodium lauryl sulphate).

Cr. No.	Test chemical	9	∕₀ Cys-pepti	de depletio	n	% Lys-peptide depletion				
Sr. NO.		+ UV	SD	- UV	SD	+ UV	SD	- UV	SD	
1	Cinnamaldehyde	72.53	0.61	72.02	0.09	60.19	1.90	56.67	2.82	
2	Chlorpromazine	99.03	0.05	0.45	0.77	9.65	1.87	0.05	0.09	
3	SDS	1.50	1.48	0.00	0.00	94.93	0.26	95.08	0.27	
4	L-Histidine	0.00	0.00	0.00	0.00	0.28	0.49	0.34	0.59	
5	Amiodarone HCI	99.71	0.01	0.00	0.00	0.06	0.13	0.84	1.32	
6	Protoporphyrin IX, Disodium	99.78	0.01	40.08	1.20	12.16	8.34	17.99	4.63	
7	Anthracene	100.00	0.00	10.28	10.59	1.57	2.71	1.80	1.58	
8	Hexachlorophene	100.00	0.00	93.26	5.87	24.24	1.91	11.83	0.24	
9	Norfloxacin	99.18	0.06	5.64	8.23	24.14	1.25	24.18	1.13	



Fig. 3: Identification of optimum parameters for photo-DPRA assay

A) CPZ and Cys- or Lys-peptides were exposed to UV, after measurement, UVA exposure time was set to have UV dose from 1 J/cm² to 5 J/cm², as shown in the graph. % peptide depletion was analyzed by HPLC-UV, after 24 ± 2 h. B) Cys-peptide and CPZ mixture was exposed to 1 J/cm² UVA and was further incubated for indicated time, followed by HPLC-UV analysis of % peptide depletion. C, D) Photo-DPRA was performed at the indicated CPZ concentrations using Cys- and Lys- (C) or only Cys- (D) peptide. E) Different concentrations of Cys-peptide as shown in the graph were mixed with 100 μ M (stock concentration) CPZ and incubated for indicated duration after 5 J/cm² UVA exposure. % peptide depletion was assessed using HPLC-UV. Note that the final CPZ concentrations are 20 times lower than the stock concentrations shown here.

3.5 Optimization of photo-DPRA using Chlorpromazine

To further optimize the photo-DPRA, we used CPZ, which produced the highest % peptide depletion upon UV exposure and tested the influence of varying assay conditions on % peptide depletion. 5 J/cm² UVA dose was used as described in NRU 3T3 PT assay. We checked if lower UVA doses will be potent in photosensitizing CPZ. A dose of even 1 J/cm² was found to be as effective as 5 J/cm² (Fig. 3A). Next, we checked the minimum incubation time required to see the photosensitizing effect of CPZ after UVA exposure. Incubation for 2 h was found to induce significant peptide depletion after 1 J/cm² UV dose (Fig. 3B). In previous experiments, 100 mM CPZ was used to investigate % peptide depletion. We also checked lower concentrations and found that as little as 1 mM CPZ was equally potent (Fig. 3C). Further, an assessment of the impact of CPZ concentrations in μ M range revealed that 90% of the Cys-peptide was depleted at 100 μ M CPZ, whereas depletion levels dropped below 40% at 10 μ M CPZ and were below 10% at 1 μ M CPZ (Fig. 3D). Note that the indicated concentrations are that of stock solutions, final concentrations of CPZ were 20 times lower. Next, we also varied the Cys-peptide concentration and monitored its % depletion at various time points (Fig. 3E). Linear correlations between peptide depletion and incubation time were observed at middle concentrations of 1.67 μ M and 3.34 μ M, whereas at lower concentration such linearity was lost after 6-8 h. Very high Cys-peptide concentrations of 6.67 μ M did not show a linear correlation.

3.6 Development of photo-ADRA to assess photo-sensitization potential of test chemicals

Using a similar approach as that of photo-DPRA, we also developed photo-ADRA for assessing photosensitization potential of test chemicals. The set up for the assay was similar to photo-DPRA, except that NAC and NAL were used instead of peptides. The standard curves for NAC and NAL are shown in Fig. S6C, S6D¹. The NAC and NAL % depletion values with and without UV exposure are shown in Table 6. In agreement with NRU 3T3 PT and photo-DPRA results, CPZ, anthracene, amiodarone HCl, and norfloxacin produced a strong increase in % depletion upon UV exposure, whereas protoporphyrin IX showed a moderate and hexachlorophene showed a subtle increase in % depletion. Histidine and SDS did not show any increase in % depletion upon UV exposure, as expected.

In order to identify photosensitizers in a robust manner and to distinguish between strong and weak sensitizers precisely, the difference between average % depletion values in the presence and absence of UV exposure was calculated and defined as Δ UV for photo-DPRA and photo-ADRA assay (Tab. 7). Δ UV serves as an indicator of the UV-mediated sensitization potential of a test chemical. In both assays, as expected, Δ UV values for strong photosensitizers (CPZ, anthracene, amiodarone HCl, and norfloxacin) were the highest, 44-56, whereas, Δ UV for protoporphyrin IX was 26-32 and hexachlorophene had Δ UV of 6-10. Note that the Δ UV values for L-histidine and SDS were below zero in both assays supporting their non-photosensitizing nature. Importantly, Δ UV values observed in photo-DPRA and photo-ADRA match closely for most of the tested compounds. Further screening of test compounds with known photosensitizing abilities will be helpful in defining threshold Δ UV value and categorization of photo-sensitizers.

Sr. No.	Test chemical		% NAC d	epletion		% NAL depletion				
		+ UV	SD	- UV	SD	+ UV	SD	- UV	SD	
1	Cinnamaldehyde	93.41	1.59	91.64	3.96	46.58	12.57	28.80	8.06	
2	Chlorpromazine	95.50	1.45	13.79	6.46	24.34	2.54	1.18	1.37	
3	SDS	0.42	0.72	0.00	0.00	0.00	0.00	0.00	0.00	
4	L-Histidine	0.00	0.00	0.00	0.00	0.18	0.32	0.11	0.19	
5	Amiodarone HCL	100.00	0.00	0.00	0.00	2.10	3.38	2.83	4.45	
6	Protoporphyrin IX, Disodium	98.01	1.06	45.37	12.30	17.95	3.88	6.90	2.42	
7	Anthracene	97.94	1.78	0.00	0.00	1.22	1.22	3.42	1.73	
8	Hexachlorophene	100.00	0.00	100.00	0.00	26.15	6.49	12.28	2.98	
9	Norfloxacin	100.00	0.00	2.74	2.59	1.94	1.89	1.75	1.48	

Tab 6: Analysis of photo sensitization of NRU 3T3 PT proficiency chemicals by photo-ADRA

Indicated test chemicals were exposed to $5J/cm^2$ UVA in presence of NAC or NAL, followed by further incubation for 24 ± 2 h at 25 ± 2.5 °C. After incubation, non-exposed (- UV) and UV exposed (+ UV) samples were analyzed by HPLC-UV. % NAC and NAL depletions were calculated as described in methods section. Values represent mean of three independent experiments with three replicates in each experiment. (Abbreviations, SDS: Sodium lauryl sulphate, SD: standard deviation).

Tab 7: Usefulness of Δ UV parameter in discrimination of photosensitizing ability of test chemical using photo-DPRA and photo-ADRA

Average % Cys + Lys-peptide and average % NAC + NAL depletions, respectively, in photo-DPRA and photo-ADRA were calculated as indicated. Difference in % depletion values with UV (+ UV) and without UV (- UV) exposure was denoted as Δ UV. Note the close match between the Δ UV values of photo-DPRA and photo-ADRA. (Abbreviations, Avg: Average, SDS: Sodium lauryl sulphate, SD: standard deviation).

0	Test Chemical	Avg % Depletion DPRA				DPRA	Avg % Depletion ADRA				ADRA
5r. No.		+ UV	SD	- UV	SD	Δυν	+ UV	SD	- UV	SD	ΔUV
1	Cinnamaldehyde	66.36	0.65	64.35	1.44	2.01	70.00	6.16	60.22	3.74	9.78
2	Chlorpromazine	54.34	0.92	0.25	0.43	54.09	59.92	0.98	7.48	3.91	52.44
3	SDS	48.21	0.61	47.54	0.14	0.67	0.21	0.36	0.00	0.00	0.21
4	L-Histidine	0.14	0.24	0.17	0.29	0.00	0.09	0.16	0.05	0.09	0.04
5	Amiodarone HCI	49.89	0.05	0.42	0.66	49.47	51.05	1.69	1.43	2.46	49.62
6	Protoporhyrin IX	55.97	4.17	29.04	2.86	26.93	57.98	1.79	26.14	5.78	31.84
7	Anthracene	50.78	1.36	6.04	5.79	44.74	49.58	0.46	1.71	0.86	47.87
8	Hexachlorophene	62.12	0.95	52.54	2.82	9.58	63.08	3.25	56.14	1.49	6.94
9	Norfloxacin	61.66	0.62	15.00	4.52	46.66	50.97	0.95	2.24	1.67	48.73

4 Discussion

Multiple alternative methods that include modification of existing tests as well as novel approaches, representing different KEs, have been proposed and are currently being evaluated to facilitate accurate skin sensitization hazard predictions. The U937 sensitization test (U-SENSTM), Interleukin-8 Reporter Gene assay (IL-8 Luc assay), and GARDTM assay analyze changes in dendritic cell biology similar to h-CLAT, whereas Lu-Sens and SENS-IS assays assess keratinocyte response much like KeratinoSensTM (Hoffmann et al., 2018; Roberts, 2018).

Similarly several *in chemico* reactivity assays that can be used to assess the MIE in the skin sensitization AOP have been reported in the literature (Aptula et al., 2006; Natsch and Gfeller, 2008; Avonto et al., 2015; Nepal et al., 2018; Zhang et al., 2018). In the present study, we compared and evaluated the utility of two alternative *in chemico* approaches, DPRA and ADRA, to support the discrimination between skin sensitizers and non-sensitizers as well as photosensitizers. Initially with the 10 proficiency substances described in the OECD 442C guideline, we used a proficiency system to investigate and compare the technical performance of DPRA & ADRA. According to OECD guidelines, these substances were selected to represent a range of responses for skin sensitization hazard and also because of the availability of high quality reference data. Notably, Cys- and Lys-peptide depletion values for the 10 proficiency compounds fell within the respective reference ranges, except for the Cys-peptide value of formaldehyde. However, this does not change the prediction outcome, as formaldehyde is predicted to be a sensitizer, as expected. As described above in the results section, inconsistent peptide reactivity with aldehydes is a common phenomenon and may be attributed to oxidation of the aldehyde group of carboxylic acid. Moreover, the test guideline also recommends that 8 out of 10 compound depletion values should fall within the respective reference ranges. Observed values matched with the reference values for 9 proficiency compounds and the predictions were accurate for 10 out of 10 compounds, indicating technical proficiency of the setup.

The DPRA assay is based on HPLC-UV detection of residual Cys- and Lys-peptides which have relatively low UV absorption. Therefore, to observe significant depletions of model peptides it is necessary to use a higher concentration of test chemicals. This limits the use of highly hydrophobic compounds, especially with the Lys-peptide. To overcome this, LC-MS methods were developed that relied on peptide-chemical adduct detection and used 1/25 test chemical concentration than that of DPRA (Natsch and Gfeller, 2008; Aleksic et al., 2009). Although more sensitive, LC-MS assays are costly to run and also show poor versatility. A stopped-flow analysis method enables use of a much lower test chemical concentration in the assay, however, an analysis performed without separation of test chemicals from peptides could compromise the detection precision (Chipinda et al., 2010). ADRA makes it possible to use 1/100th test chemical concentration than that of DPRA and is also cost effective as it uses HPLC-UV for analysis. Analysis at 281 nm, as opposed to 220 nm, significantly improves the baseline stability in ADRA (Fujita et al., 2014). Recent studies also suggested use of EDTA in ADRA significantly reduces oxidation of the thiol group, without affecting prediction accuracy (Fujita et al., 2018).

In the light of the described ADRA advantages, we carried out comparative analysis of ADRA and DPRA. Data suggested both methods predict and discriminate between sensitizers and non-sensitizers, equally well. Although we used a limited number of chemicals, the assays had high concordance within their predictions. We suggest that the ADRA and DPRA could be used as alternative methods whenever applicability of one of them is limited by solubility, precipitation and co-elution of test chemical or any

other technical issue, such as incompatible reaction buffer pH, etc. Both assays provided the same predictions for 25 tested chemicals, which matched with previously published data, except for vanillin. Although, vanillin is a known *in vivo* non- sensitizer, multiple studies reported contradictory in *chemico* and *in vitro* data (Basketter et al., 2001; Basketter and Kimber, 2010). This may be due to the reduced reactivity of a vanillin's para hydroxyl benzaldehyde group to form a Schiff base, compared to other aldehydes (Natsch et al., 2012).

Prior to implementation of 3R's principles, tests employing rabbits, guinea pigs, rats, and mice were commonly used to evaluate photoirritation and photoallergy, as well as sensitization. The International Conference on Harmonization (ICH) has developed a S10 test guideline for preclinical and clinical photosafety assessment of pharmaceuticals. This guideline is recommended by regulatory bodies in Europe, Japan, Canada, and USA (Food and Drug Administration, HHS, 2015). More recently, non-animal *in vitro* alternatives such as ITS strategies and the NRU 3T3 PT assay have been adopted by regulatory bodies to predict the sensitivity and phototoxicity of test chemicals, respectively (OECD, 2004; Prior et al., 2019). A similar well-defined approach to assess the photosensitizing potential of test chemicals using *in vitro* methods is not yet available. Here, we showed that modification of the DPRA and ADRA by the introduction of a UVA irradiation step is capable of robustly predicting the photosensitizer nature of chemicals. We evaluated test chemicals described in the OECD 432 NRU 3T3 PT assay in photo-DPRA and photo-ADRA. A high correlation between *in vivo* test outcome and NRU 3T3 PT assay predictions have been well established (Ceridono et al., 2012). UV-VIS light spectra analysis to check the light absorption pattern as well as measurement of singlet oxygen using ROS (reactive oxygen species) assays has been suggested to evaluate the photosensitive nature of test chemicals (Nishida et al., 2015). However, these are indirect approaches that do not address the MIE or any KE involved in sensitization AOP.

Based on the difference in depletion values of a compound in DPRA/ADRA and Photo-DPRA/Photo-ADRA, we defined a parameter called ΔUV . Higher ΔUV values correlated with higher photo-sensitizing ability of a compound. Higher ΔUV value denotes the increased peptide depletion in the presence of UV. Interestingly, compounds such as CPZ, amidarone HCl and anthracene, which are non-sensitizers, could be accurately predicted as photo-sensitizers using photo-DPRA and photo-ADRA, indicating their pre-hapten nature. Further, these assays could potentially discriminate between the photo-sensitizing potential of strong and moderate photosensitizers such as CPZ and protoporphyrin IX, respectively. A close match between ΔUV values in both assays for strong photo-sensitizers (54.09 v/s 52.44 CPZ, 49.47 v/s 49.62 for amidarone HCl, 44.74 v/s 47.87 for anthracene and 46.66 v/s 48.73 for norfloxacin) as well as similar predictions of moderate, weak and non-photosensitizers strongly support the accuracy of the ΔUV for identifying photoallergens. During the course of these studies, another study reporting a use of similar approach to investigate the photosensitivity of glyphosphate containing pesticides was published by another group (de Ávila et al., 2017). Their data clearly indicated the suitability of photo-DPRA in identification of photosensitizers. ΔUV values calculated from their % peptide depletion data for protoporphyrin IX and for amidarone HCl are 22.16 and 43.57, respectively. Interestingly, these values are close to the ΔUV values obtained in our photo-DPRA study. However, a third common test chemical used in both studies, hexachlorophene, did not have concordant ΔUV values. Note that % depletion values for hexachlorophene in our hands showed high experimental variations, which needs further investigation. Independent evaluations of such modified assays using diverse test chemicals belonging to weak, medium, high, or non-photosensitizer categories is critical to promote its acceptance. Moreover, as indicated by photo-DPRA data using CPZ, further optimization of assay conditions, such as UV exposure dose, time, etc. is necessary to use these methods efficiently and in a cost effective manner.

KEs involved in the photosensitization pathway are essentially the same as that of sensitization AOP, except that there is an extra triggering event (TE) that involves interaction of light with the test chemical and its excitation to higher energy level, followed by a common MIE that represents cross-linking of chemical with protein (haptenization) and subsequent KEs. Thus, in order to evaluate the photosensitivity of a test chemical, adopting a weight-of evidence approach similar to one that is used to address sensitization, with inclusion of a robust assay to address the TE would be an ideal approach. Photo-DPRA and photo-ADRA are highly suitable methods to analyze the TE in the photosensitization AOP, an additional advantage of such an approach will be the analysis of TE and MIE in the same assay. This will reduce the use of animals in cosmetic/ drug testing and will also be able to avoid confusions that are common in the diagnosis of drug induced photosensitization in clinical set ups (Lugović-Mihić et al., 2017). *In vitro* test methods that employ UVA exposure of NCTC2544 Keratinocytes (Galbiati et al., 2013) and THP-1 monocytes (Hoya et al., 2009; Martínez et al., 2013) to detect IL-18 and IL-8, CD86 and CD54, respectively, and predict keratinocyte/ dendritic cell activation and subsequent photosensitization has been described in the literature. This further supports the feasibility of developing an integrated strategy to evaluate photosensitizers.

Overall results show at par performance of ADRA and DPRA in discriminating skin sensitizers from non-sensitizers. Similarly, results also support equal efficiency of photo-DPRA and photo-ADRA in analysis of the photosensitizing potential of test chemicals. Careful repetition and validation of ADRA as well as photo-DPRA and photo-ADRA across laboratories is necessary to underscore their utility. Although, a limited number of chemicals were tested, the pronounced similarity of Δ UV values in photo-DPRA and photo-ADRA, as well as an effective identification of photosensitizers with these assays, is highly promising. Similar to DPRA, validation and adoption of ADRA, photo-DPRA and photo-ADRA by regulatory bodies will be immensely helpful in advancing the use and acceptance of these non-animal alternative methods.

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Conflict of interest

The authors declare that they have no conflicts of interest.