The Assessment of Phototoxicity Using the 3T3 Neutral Red Uptake (NRU) Phototoxicity Assay and a Modified Photo-Direct Peptide Reactivity Assay (DPRA)

Gamson, A., Herpel, T., Lamm, M., Sadowski, N., Sheehan, D., Hilberer, A.
Institute for *In Vitro* Sciences, Inc., Gaithersburg, MD USA

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Introduction

Alternative methods, including the validated 3T3 Neutral Red Uptake (NRU) Phototoxicity assay (OECD TG 432) may be used as a pre-clinical test to address phototoxicity. Currently, there are no validated alternative test methods to identify photoallergens; however, there are several validated alternative test methods to address skin sensitization, including the Direct Peptide Reactivity Assay (DPRA) (OECD 442C). To address photoallergy, we performed the standard DPRA with a modification to include a UVA exposure (5 J/cm^2) as described by Hayato, et al. (2016). The Dermalight SOL 3 solar simulator equipped with H1 filter (320-400 nm) was used for all photo-irradiation. The amount of cysteine (Cys) peptide depletion was monitored immediately after UVA/dark exposures (time 0) and at 2 hour intervals up to a 26 hour period (See Figure 3). We utilized the 3T3 Phototoxicity assay (Figure 1a) in combination with a modified photo-DPRA assay (Figure 1b) to determine if these assays were able to 1) identify compounds with phototoxicity potential and 2) discriminate between photoirritants and photoallergens.

Materials & Methods

To establish proof of concept, we selected a small subset of seven compounds, identified as: photoirritants and photoallergens [chlorpromazine (chlor), 6-methylcoumarin (6-MC), and amiodarone], photoallergen (hexachlorophene), photoirritant (anthracene), allergen [cinnamic aldehyde (CA)], and non-allergen and non-photoirritant [lactic acid (LA)]. For the 3T3 Phototoxicity Assay, the Phototox 2.0 software was used to calculate the Mean Photo Effect (MPE), or briefly, the difference in responses of cells exposed to the test compound in the presence and absence of UVA/visible light. Representative dose response curves are presented in Figure 2. For the photo-DPRA, the Empower 3 software was used to calculate the Area Under the Curve (AUC) for determination of peptide depletion by each test compound exposed in the presence and absence of UVA/visible light. The % depletion for each compound exposed in the presence of UVA (dotted lines) and absence of UVA (solid lines) over a time course is presented in Figure 3. Two times, time 0 (i.e., immediately after UVA/dark exposure) and 24±2 hours post exposure (for comparison to the standard DRPA assay which allows a 24±2-hour reactivity time), were selected to show the difference in peptide depletion between UVA and dark-exposed reactions (Figure 4).

To ensure that the UVA exposure didn't affect the Cys peptide, the peptide was exposed to 12 J/cm2 of UVA exposure and showed 0% or 1.0% depletion (time 0 or 26 hours), indicating peptide stability after UVA exposure.

The clinical classification of each compound (photoallergen and/or photoirritant) was assigned using references cited from Ahmad et al. (2016), Maibach & Honari (2014), and Onoue et al. (2017). The phototoxicity potential was evaluated in the 3T3 Phototoxicity Assay by the Mean Photo Effect (MPE) values. A compound was predicted to have phototoxicity if the MPE was >0.150 and no phototoxicity if the MPE was <0.100 (OECD TG 432). The peptide depletion cut-offs described in OECD TG 442C were used as guidance (i.e., peptide depletion > 13.89% was considered to be positive for sensitization potential). A summary of the results for each compound is presented in Table 1.

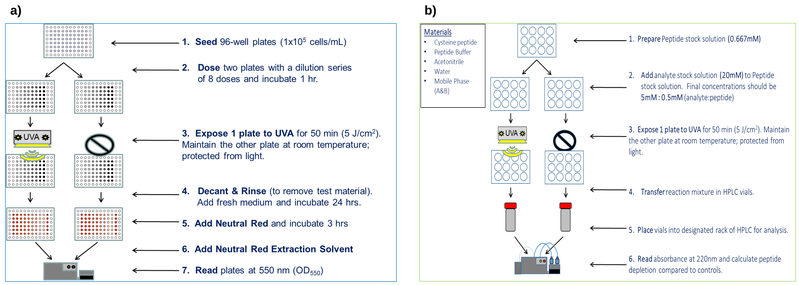

Figure 1. Step by step procedures for a) the 3T3 Phototoxicity Assay and b) the Photo-DPRA assay

References

- Maibach H. & Honari G. (2014) Applied Dermatotoxicology: Clinical Aspects. Copyright Elsevier Inc.
- Onoue et al. Chemical Photoallergy: Photobiochemical Mechanisms, Classification, and Risk Assessments. Journal of Dermatological Science. 85 (2017) 4-11
- Ahmad I, et al. Photostability and Photostabilization of Drugs and Drug Products. International Journal of Photoenergy. Vol. 2016, Article ID 8135608
- Hayato Nishida, Takao Ashikaga, Morihiko Hirota, Satomi Onoue, Yoshiki Seto, and Hirokazu Kouzuki. Development of photo Direct Peptide Reactivity Assay. Presented at SOT 2016 Annual Meeting, New Orleans, LA
- OECD TG 432: *In Vitro* 3T3 NRU Phototoxicity Test (adopted 13 April 2004)
- OECD TG 442C: *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA) (adopted 4 February 2015)

Results

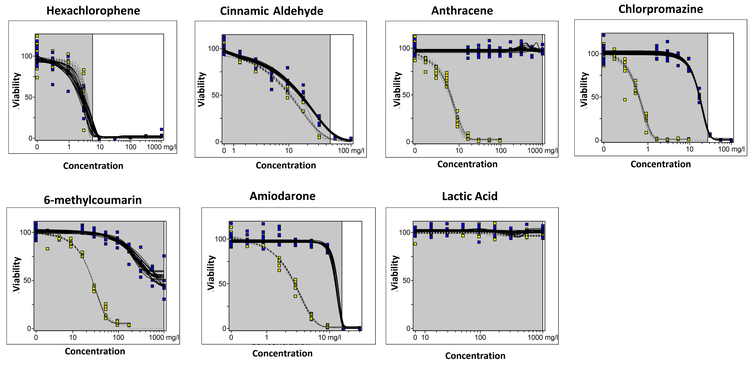

Figure 2. Selected dose response curves from the 3T3 Phototoxicity Assay. The concentrations chosen were based on the highest testing limits of the OECD TG 432 (*i.e.*, 1000 µg/mL) or based on the cytotoxicity profile of a dose range finding assay. The relative viability was calculated over tested concentrations in the presence (yellow boxes) and absence (blue boxes) of UVA. Each yellow or blue box represents relative viability of an individual well at each concentration (6 total replicates per UVA or dark exposure). Area shaded in grey was used to calculate the MPE value.

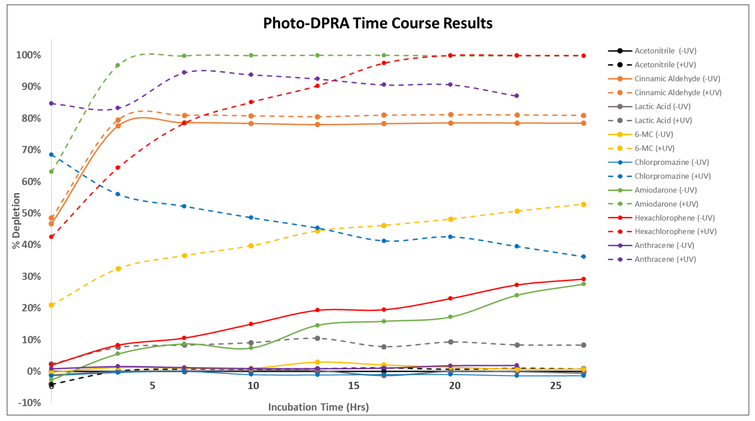

Figure 3. Photo-DPRA time course reactivity response of test compounds in the presence (+UV) and absence (-UV) of UVA/visible light. After UV-irradiation or dark exposure, the depletion of cysteine (Cys) peptide was determined (time 0), and at 2 hour intervals thereafter for up to 26 hours. Dotted lines represent Cys depletion for each test compound after exposure to UVA (+UV) and solid lines represent Cys depletion for each test compound exposed in the dark (-UV).

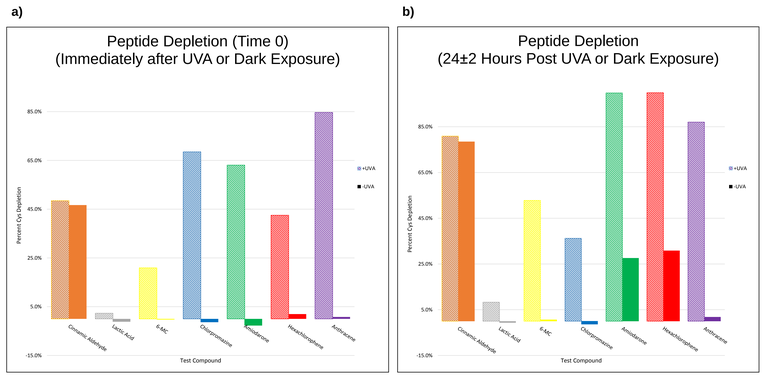

Figure 4. Representative results of the photo-DPRA time course assay with differences in Cys peptide depletion immediately after 5 J/cm^2 UVA (+UV) or dark (-UV) exposure (time 0) (a) and 24±2 hours after UVA or dark incubation with test compound (b). The striped bars represent the percent peptide depletion after exposure to UVA; the solid bars represent the percent peptide depletion of the test compounds exposed in the dark (-UVA).

Test Compound	MPE	Average Depletion[1] (+UVA)	Average Depletion[1] (-UVA)	Classification *In Vitro*	Classification Clinical[2]
6-methylcoumarin	0.402	21.0%	-0.3%	Photoirritant, Photoallergen	Photoirritant, Photoallergen
Amiodarone	0.284	63.1%	-2.7%	Photoirritant, Photoallergen	Photoirritant, Photoallergen
Anthracene	0.749	84.7%	0.8%	Photoirritant, Photoallergen	Photoirritant, Non-Photoallergen
Chlorpromazine	0.611	68.5%	-1.4%	Photoirritant, Photoallergen	Photoirritant, Photoallergen
Hexaclorophene	-0.019	42.5%	2.0%	Non-Photoirritant, Photoallergen	Non-Photoirritant, Photoallergen
Cinnamic Aldehyde	0.011	48.5%	46.7%	Non-Photoirritant, Non-Photoallergen	Non-Photoirritant, Non-Photoallergen
Lactic Acid	-0.018	2.3%	-1.2%	Non-Photoirritant, Non-Photoallergen	Non-Photoirritant, Non-Photoallergen

[1]- Average depletion of peptide (at least 3 trials) immediately after UV/dark exposure (time 0)

[2]- Clinical classification as described in Ahmad, Maibach, and/or Onoue

Table 1. Summary results for 3T3 Phototoxicity Assay (MPE values) and photo-DPRA (% depletion of Cys peptide for each compound in the presence (+UV) and absence (-UV) of UVA at immediately after UVA or dark exposure (time 0)). Classification of each compound using results from alternative test methods (*i.e., in vitro*) and clinical classification using referenced literature (as referenced in Ahmad, et al. (2016), Maibach & Honari (2014) and/or Onoue et al. (2017)). Positive responses for photoirritation and photoallergy in red; negative responses in green; and mixed responses (*e.g.* photoirritant and non-photoallergen or non-photoirritant and photoallergen) in green-red boxes.

Conclusions & Future Directions

- All compounds identified as phototoxic in the 3T3 NRU Assay showed differences (≥21.0%) in reactivity in the presence of UVA as compared to the absence of UVA
- Hexachlorophene, which did not trigger a phototoxic response in the 3T3 NRU assay (*i.e.,* MPE <0.100), would have been correctly identified as a photoallergen in the Photo-DPRA
- The peptide depletion in the presence of UVA was very high (>80%) for Anthracene, a photoirritant, throughout the time course (up to 24 hours) of the Photo-DPRA
- Although we were not able to discriminate between photoirritants and photoallergens, our tiered testing approach was able to identify compounds which posed a phototoxic hazard (e.g. photoirritant and photoallergen)
- Over the time course we noted increases in peptide depletion for some compounds in the absence of UVA, more notably as the reactivity time neared 24 hours
- Future Directions: Investigation in the use of other validated skin sensitization methods with a photo-irradiation modification, as well as a RhE model; evaluation of a larger subset of compounds; and use of lysine peptide