Evaluation of New Solvents for the Use in the Multi-Dose Reconstructed Human EpiDermis (RhE) Phototoxicity Assay

D. Sheehan, A. Pidathala, A. Hilberer

Institute for In Vitro Sciences, Inc. (IIVS), Gaithersburg, MD, USA

ABSTRACT

The phototoxic potential of test materials after exposure to UVA/visible light is evaluated by the 3T3 Neutral Red Uptake Phototoxicity Assay using Balb/c 3T3 mouse fibroblasts (OECD TG 432). To address challenges related to testing of finished products or materials that are not completely soluble, the Reconstructed Human EpiDermis (RhE) Phototoxicity Assay (INVITTOX Protocol 121) can be used as a stand-alone or in a tiered approach. Water and sesame oil were recommended solvents for the RhE Assay, however additional solvents may be investigated on a case-by-case basis to accommodate a wider variety of test materials. Alternate solvents should first be assessed to ensure that they: 1) do not cause cytotoxicity; 2) do not diminish and/or inhibit phototoxic reactions; and 3) do not interfere with the UVA exposure. We investigated 5% DMSO in Hanks' Balanced Salt Solution (HBSS), 5% acetone in HBSS, and polyethylene glycol (PEG) and their influence on the prediction of phototoxicity of chlorpromazine, a known phototoxicant. Duplicate tissues (EpiDerm™ from MatTek Corporation, Ashland, MA) were treated with each group for 24 hours, followed by a UVA (~6 J/cm2) or dark exposure, and then a 21 hour post-exposure period before viability assessment using MTT dye. The assay positive control, 0.02% chlorpromazine in HBSS containing 1% DMSO, was tested concurrently. A test material was considered to have phototoxic potential if it induced a ≥30% difference in viability between tissues exposed to UVA as compared to the dark-exposed tissues. Our data showed that the solvents performed in a comparable manner to the assay negative control (HBSS) and they did not induce significant toxicity when the viability of the UVA-exposed and dark-exposed tissues was compared: 92.9% and 104.8% (5% DMSO), 94.0% and 97.5% (5% acetone), and 81.3% and 90.0% (PEG), respectively. The difference between the viability of the UVA-exposed or dark-exposed tissues after treatment with 0.02% chlorpromazine dissolved in 1% DMSO, in 5% DMSO, and in PEG was 60.0%, 59.7%, and 72.5%, respectively. These data indicate that the new solvents we investigated were suitable for use in the detection of phototoxicity. The evaluation of 0.02% chlorpromazine in 5% acetone, as well as additional solvents (e.g. ethanol), is currently ongoing. Our future investigations will concentrate on the assessment of different solvents that can accommodate the phototoxicity testing of novel chemistries.

INTRODUCTION

Phototoxicity or photoirritation is a toxic response that is elicited after the exposure of skin to certain chemicals and subsequent exposure to light. If a chemical absorbs UV or visible light, photosafety assessments may be warranted to determine if the test material is likely to cause adverse effects after exposure to light. Therefore, the identification of test materials (ingredients and/or formulations) able to elicit a phototoxic reaction is a crucial step in risk assessment processes. The 3T3 Neutral Red Uptake (NRU) Phototoxicity assay is an established *in vitro* assay used to evaluate the potential phototoxicity hazard of a test material (OECD Test Guideline 432). However, limitations exist in the dilution based, monolayer culture test system that may be addressed using a more complex model where solubility is not a limiting factor and a wider variety of test materials (e.g. formulations) may be evaluated. The use of a reconstructed human epidermis (RhE) EpiDerm™ model is an alternative testing platform able to overcome these limitations. The EpiDerm™ model is composed of normal human-derived epidermal keratinocytes stratified to form multiple layers, including a functional *stratum corneum*. With the inclusion of the *stratum corneum,* this model mimics human skin and provides a barrier function. RhE models allow for the topical application of a wide variety of test materials of differing physicochemical properties, including water insolubles, extreme pH values, ingredients, and finished products or complex formulations.

Although a formal validation was not conducted, pre-validation efforts for the RhE models were underway in the late 1990s and showed promising potential for use in photosafety assessments. Through the efforts of ZEBET and others, an INVITTOX Protocol (Number 121) was published and used as guidance for conducting the procedures detailed in our work. The promising performance of this assay encouraged us to look into areas for optimization and development to suit a wider range of test materials.

We utilized the EpiDerm™ model (MatTek Corporation) to examine the cytotoxic and phototoxic potential of the prospective assay solvents, including PEG, DMSO, acetone, and ethanol. Tissue viability was determined using the MTT conversion assay. Table 1 summarizes the responses of each solvent, relative to the exposure-matched HBSS, after exposure in the presence and absence of UVA light. The responses are graphically represented in Figure 1. The 0.02% chlorpromazine was evaluated in the prospective solvents after exposure in the presence and absence of UVA light to determine if solvent was suitable for detection of phototoxic potential. As outlined in the INVITTOX Protocol 121 and IIVS' protocols, a treatment was considered to have exhibited phototoxic potential if viability of the tissues exposed in the presence of UVA showed a difference of ≥30% as compared to the tissues exposed in the absence of UVA. Table 2 summarizes the responses of the chlorpromazine in each respective solvent, as well the difference in viability of the tissues exposed in the presence of UVA compared to the absence of UVA. The responses were graphed in Figure 2. Each trial included the assay positive control, 0.02% chlorpromazine diluted in HBSS containing 1% DMSO, and was used as a comparison. Each solvent was evaluated in at least one trial, and four trials were conducted for the positive control (average results presented). An example of a dose response approach using chlorpromazine is shown in Figure 3.

REAGENTS

- EpiDerm™ Tissues (EPI-200). MatTek Corporation (Ashland, MA, USA)
- EpiDerm™ Assay Medium (ASY-100). MatTek Corporation (Ashland, MA, USA)
- MTT (3-[4,5 - dimethylthiazol-2-yl] - 2,5 - diphenyltetrazolium bromide) (Catalog# M5655). Sigma (St. Louis, MO, USA)
- Phosphate Buffered Saline, pH 7.4 (Catalog# 114-058-101) used to prepare stock 10 mg/mL MTT. Quality Biologicals (Gaithersburg, MD, USA)
- Dulbecco's Modified Eagle's Medium (DMEM), high glucose (Catalog# 112-013-101) supplemented with 2 mM L-glutamine, used to prepare 1 mg/mL MTT. Quality Biologicals (Gaithersburg, MD, USA)
- Ca^{++} and Mg^{++} Free Dulbecco's Phosphate Buffered Saline (CMF-DPBS) (Quality Biologicals, Gaithersburg, MD, USA)
- Chlorpromazine Hydrochloride (Catalog# C8138). Sigma (St. Louis, MO, USA)
- Dimethyl Sulfoxide (DMSO), Chromasolv® Plus ≥99.7% (Catalog# 34869). Sigma (St. Louis, MO, USA)
- Polyethylene glycol (PEG), 200 MW (Catalog# P3015). Sigma (St. Louis, MO, USA)
- Ethanol (EtOH), 200 Proof Anhydrous Reagent Alcohol (Catalog# 241000200). Pharmco (Brookfield, CT, USA)
- Acetone, Chromasolv® Plus ≥99.9% (Catalog# 650501). Sigma (St. Louis, MO, USA)
- Isopropanol, ACS Reagent ≥99.5% (Catalog# 190764). Sigma (St. Louis, MO, USA)
- Penicillin (10,000 IU/mL)/Streptomycin (10,000 µg/mL) (Catalog# 120-095-721). Quality Biologicals (Gaithersburg, MD, USA)
- Hanks' Balanced Salt Solution (HBSS) (Catalog# 14025-092). Gibco/Life Technologies (Grand Island, NY, USA)

Table 1. Summary of Solvent Relative Viability

Solvent	% Relative Viability*	
	+UVA	-UVA
PEG	81.3	90.0
5% DMSO in HBSS	92.9	104.8
5% Acetone in HBSS@	100.9	103.6
5% Ethanol in HBSS	103.8	97.6
1% DMSO in HBSS	107.5	106.0

**- % viability relative to +UVA or -UVA HBSS*
@- Tested in 2 trials (average viabilities presented)

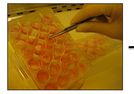
Table 2. Summary of 0.02% Chlorpromazine in Solvents

0.2% Chlorpromazine in Solvent	% Relative Viability*		Difference Between +UVA & -UVA
	+UVA	-UVA	
PEG	19.4	91.9	72.5
5% DMSO in HBSS	35.5	95.2	59.7
5% Acetone in HBSS	25.3	97.3	72.0
5% Ethanol in HBSS	35.0	101.9	66.9
1% DMSO in HBSS^	28.6	94.5	65.9

**- % Viability relative to matched +UVA or –UVA solvent control*
^- Assay Positive Control; Average viabilities of 4 trials presented

EXPERIMENTAL DESIGN

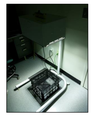
Tissue Receipt & Equilibration
Tissues, received on agarose, were transferred to 6-well plates containing culture medium and incubated for at least 1 hour at standard culture conditions to equilibrate the tissues.



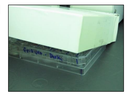
Tissue Dosing
Tissues dosed in quadruplicate with 50 µL of each treatment group (assay positive control, chlorpromazine in solvent, solvent control, or chlorpromazine concentration).

24 ± 1 hours

Rinsing & Transfer to 24-well Plates
Tissues rinsed with sterile DPBS to remove treatments and then the tissues were transferred to 24-well plates (designated as +UVA or –UVA) containing HBSS in preparation of the UVA/dark exposures.

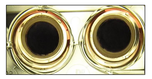


UVA and Dark (-UVA) Exposure
Half of the tissues from each treatment group were transferred to the HBSS plate designated for the UVA exposure and half were transferred to an HBSS plate retained in the dark for a 1 hour exposure. The SOL3 Dermalight Solar Simulator (UvaTec), equipped with an H1 filter was used to irradiate the tissues for 1 hour, resulting in a total exposure of total of 6 J/cm². After the UVA/dark exposures, the tissues were transferred into fresh culture medium and incubated at standard culture conditions.



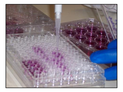
21 ± 1 hours

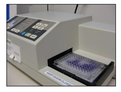
MTT Incubation
After the incubation, the tissues were transferred to 1 mg/mL MTT and incubated at standard culture conditions.



3 ± 0.1 hours

MTT Extraction & Optical Density Determination
The tissues were transferred into 2 mL of isopropanol to extract the MTT. 200 µL aliquots were transferred into 96-well plates and the amount of MTT extracted was quantified using a spectrophotometer.





DATA ANALYSIS & PREDICTION MODEL

$$\% \text{ Viability} = \frac{\text{corrected } OD_{550} \text{ of UVA/Dark Exposure Matched Treatment Group}}{\text{corrected } OD_{550} \text{ of UVA/Dark Exposure Matched Solvent Control}} \times 100$$

If the treatment induced ≥30% decrease in viability in the presence of UVA compared to the viability in the absence of UVA, then the treatment was considered to have exhibited a phototoxic response.

CONCLUSIONS & CONSIDERATIONS

- All prospective solvents showed minimal (if any) cytotoxicity with relative viabilities remaining >80% for tissues exposed in the presence and absence of UVA/visible light
- All prospective solvents showed minimal decreases in viability in the presence of UVA as compared to the absence of UVA, indicating that the solvents themselves did not exhibit phototoxicity
- Chlorpromazine (diluted at 0.02%) was correctly predicted as phototoxic (per prediction model of >30% difference) in all prospective solvents, with all prospective solvents showing similar responses of the tissues exposed in the presence of UVA as compared to the absence of UVA. These results demonstrated that the solvents did not quench or enhance the phototoxic potential of the chlorpromazine
- 0.02% chlorpromazine diluted in all of the solvents performed similarly to the assay positive control (0.02% chlorpromazine in HBSS containing 1% DMSO)
- Our results demonstrated that the prospective solvents tested can be considered for use in photosafety assessments using the RhE EpiDerm™ model
- Potential solvents should be evaluated prior to use to demonstrate proper assay performance. We have outlined a strategy to determine appropriateness of novel solvents for use in photosafety assay using the RhE model

RESULTS

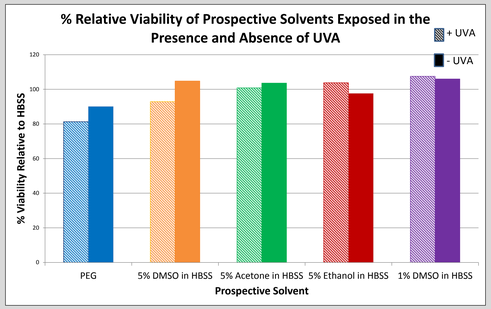


Figure 1
Cytotoxicity of Alternate Solvents

The cytotoxicity of each solvent was assessed relative to the exposure-matched HBSS (+UVA or –UVA). The prospective solvents were tested at 5% in HBSS, with the exception of PEG, which was tested without dilution. This figure compares the viability of these solvents in the presence (+UVA) and absence (-UVA) of UVA.

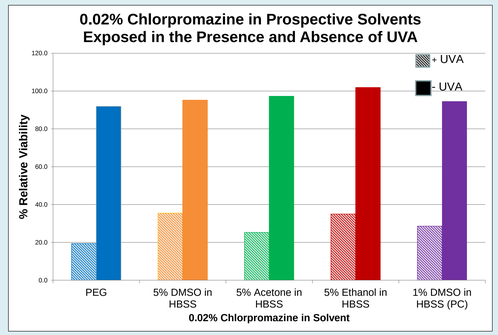


Figure 2
Phototoxicity of Chlorpromazine in Alternate Solvents

Responses of 0.02% chlorpromazine diluted in each prospective solvent and then exposed in the presence (+UVA) or absence (-UVA) of UVA. The average relative viability of the assay positive control (PC) (0.02% chlorpromazine diluted in 1% DMSO in HBSS) over 4 trials was shown for comparison.

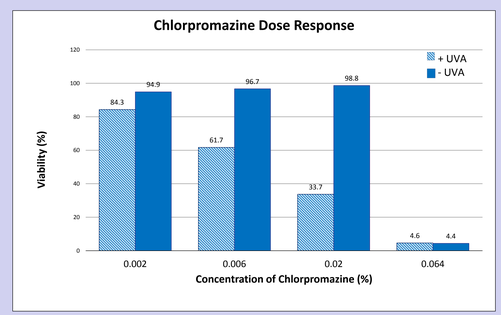


Figure 3
Serial Dilution of Chlorpromazine

Dose responses of the chlorpromazine serially diluted in 1% DMSO in HBSS using a dilution factor of ~3.2 (~1/2 log steps). The tissues were treated with 0.002%, 0.006%, 0.02%, and 0.064% chlorpromazine for 24±1 hours. The assay positive control was (0.02% chlorpromazine).

REFERENCES

Liebsch, M. (2000) Prevalidation of the EpiDerm™ Phototoxicity Test. Final Report on Subcontract No. PHOT01

ZEBET Standard Operating Procedure EpiDerm™ Phototoxicity Assay (model:Epi-200), 5 November 1997

INVITTOX Protocol 121. EpiDerm™ Phototoxicity Assay. ECVAM DB-ALM; 1999. *http://ecvam-dbalm.jrc.ec.europa.eu/*

Acknowledgments: IIVS would like to thank Norah Sadowski, B.S. for her laboratory contributions to this R&D work