The Assessment of Alternate Solvents for Use in the 3T3 Neutral Red Uptake (NRU) Phototoxicity Assay and the Influence of Solvents on the Prediction of Phototoxic Potential of Amiodarone

Sadowski, N; Sheehan, D; Sparks, J ; Sly, E; Hilberer, A
Institute for In Vitro Sciences, Inc., Gaithersburg MD, USA

INTRODUCTION

The 3T3 NRU Phototoxicity Assay is a photo safety assay used to assess the phototoxic hazard potential of a test material. The assay procedures performed were similar to those outlined in the Organization for Economic Cooperation and Development (OECD) test guideline (TG) 432: In Vitro 3T3 NRU Phototoxicity Test (*Figure 1*). Two measures of phototoxicity are described in the TG 432: the Photo Irritancy Factor (PIF) (compares IC_{50} values, or concentration of 50% inhibition of neutral red uptake, of cultures exposed in the presence or absence of UVA) and Mean Photo Effect (MPE) (compares the dose responses of the cultures treated in the presence and absence of UVA) (*Table 1*). We evaluated the potential phototoxicity and cytotoxicity of various solvents that could be used to dissolve particularly insoluble test materials. Any solvent that passed the initial screening was used to prepare a dilution series of a known phototoxic material, chlorpromazine. The assay positive control, chlorpromazine diluted in DMSO, was tested in parallel for comparison. We evaluated the solubility, cytotoxicity, and phototoxicity potential of Amiodarone, one of the proficiency materials listed in TG 432, in various solvents. An approximate concentration where full solubility was achieved was determined for each solvent. In order to comply with the concentration limits of TG 432, the highest maximum concentration of 1 mg/mL was attempted in a dose range finding assay. At least one definitive assay was then performed using narrower dose ranges. Dilutions where precipitates were observed were not avoided, such as to better understand their impact, if any, on the test results. Amiodarone was correctly predicted by PIF and/or MPE values using intermediate solvents. When HBSS was used as the solvent, the phototoxic potential was not correctly predicted, the dose responses were not consistent across trials, and variability in responses was observed. Our data suggests that the initial screening of novel solvents is necessary prior to use in the 3T3 NRU phototoxicity assay. In addition, solvent selection is critical in the determination of the phototoxic potential of test materials.

MPE	PIF	Prediction[1]
<0.1	<2.0	Non Phototoxic
0.1≤MPE<0.150	2.0≤PIF<5.0	Probable Phototoxicity
MPE≥0.150	PIF≥5.0	Phototoxic

Table 1. Predictions of Phototoxicity based on OECD TG 432[1]

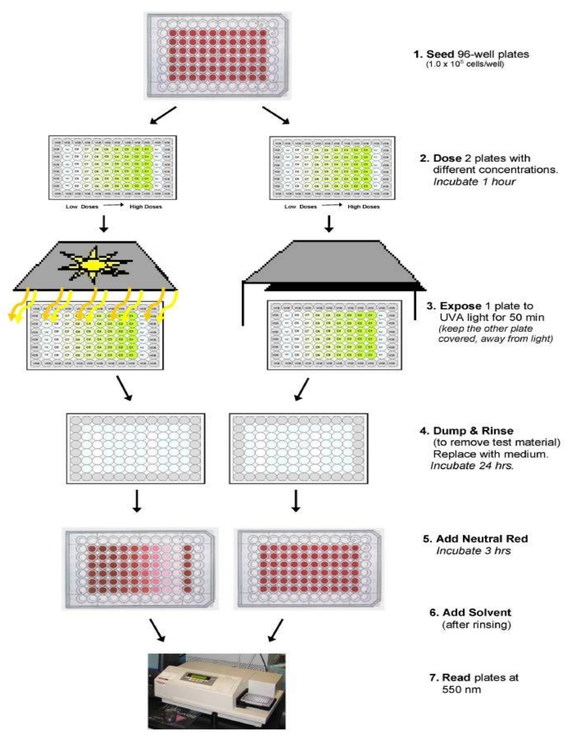

Figure 1. Generalized Overview of 3T3 Neutral Red Uptake (NRU) Phototoxicity Assay

EXPERIMENTAL DESIGN

Initial Solvent Screening

- To investigate the use of additional solvents, two 96-well plates (one for UVA exposure and one for dark exposure) were treated with various solvents dissolved in HBSS (eight concentrations ranging from 10% to 0.37%).
- Acceptability of solvents for future use was assessed by comparing dose response curves and MPE values. (*Figure 2*)
- Any solvent that was deemed suitable in the assay underwent further testing. Those solvents that were not suitable were no longer used.

Chlorpromazine in Various Solvents

- Solvents that were shown to have no adverse effects in the initial screening assay were further tested using the positive control, chlorpromazine, to ensure the solvent did not quench or enhance the phototoxicity potential.
- Chlorpromazine was diluted in the solvent (concentrations ranging from 100 to 0.156 µg/mL). One 96-well plate (+UVA) was treated with eight concentrations of chlorpromazine (9.53 to 0.156 µg/mL). The other 96-well plate (-UVA) was treated with eight concentrations of chlorpromazine (100 to 1.63 µg/mL).
- Results from chlorpromazine in alternate solvents were compared to the positive control diluted in DMSO and to IIVS' historical database range. The MPE and PIF values for each solvent were compared. (*Table 2)*

Amiodarone in Various Solvents

- Amiodarone was prepared in each solvent with concentrations selected from the preliminary screening assay to capture the toxic responses to amiodarone. One plate for UVA (+UVA) exposure and one plate kept in the dark (-UVA) were treated with eight concentrations of amiodarone.
- Results from amiodarone in alternate solvents were assessed by comparing the MPE and PIF values for each solvent. (*Table 3*)

RESULTS

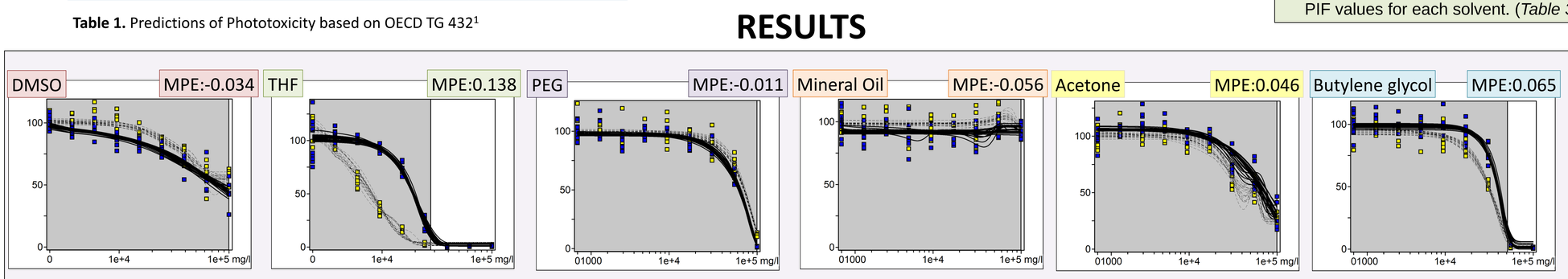

Figure 2. Dose response curves and MPE values for various solvents assessed for compatibility in the 3T3 NRU Phototoxicity Assay. Dose response curves for six different solvents prepared in HBSS (primary solvent) (concentrations ranging from 0.37% to 10%). The data were generated using Phototox 2.0 software (ZEBET). The relative viability was plotted against the concentration of the solvent. The +UVA dose responses are represented by the yellow boxes; -UVA dose responses are represented by the blue boxes. Tetrahydrofuran (THF) and Mineral Oil were not acceptable solvents for further testing.

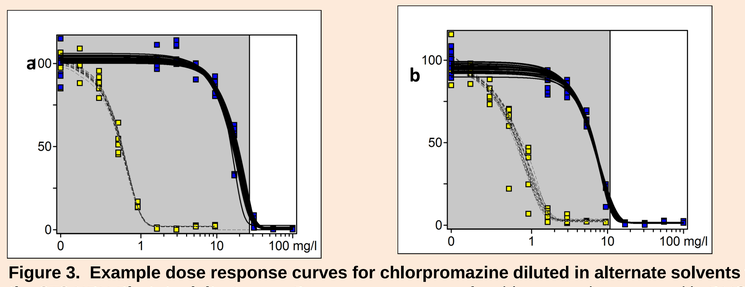

Figure 3. Example dose response curves for chlorpromazine diluted in alternate solvents for compatibility in the 3T3 NRU Phototoxicity Assay. Dose response curves for chlorpromazine prepared in DMSO (a) and HBSS (b). Concentrations ranged from 0.156 µg/mL to 100 µg/mL. The data were generated using Phototox 2.0 software (ZEBET). The relative viability was plotted against the concentration of chlorpromazine. The +UVA dose responses are represented by the yellow boxes; -UVA dose responses are represented by the blue boxes.

Solvent	MPE	PIF	Phototoxic Prediction
Poly(ethylene) glycol (PEG)	0.681	55.7	Phototoxic
Butylene glycol	0.682	45.6	Phototoxic
Ethanol	0.553	28.1	Phototoxic
Acetone	0.700	27.4	Phototoxic
DMSO	0.642	27.6	Phototoxic
HBSS	0.387	9.66	Phototoxic

Table 2. Phototoxicity results (MPE and PIF values) for chlorpromazine dissolved in various solvents. Dilutions of the positive control, chlorpromazine, were prepared in a variety of solvents and treated on two 96-well plates, one for light exposure (+UVA) and one for dark exposure (-UVA). The PIF and MPE values were calculated using the Phototox 2.0 software. The dilution of chlorpromazine in HBSS did not fall within IIVS' current historical data range (MPE values: 0.398-0.733).

Solvent	MPE	PIF	Phototoxic Prediction	Concentration Ranges for Definitive Assays (µg/mL)	Concentration where Solubility Achieved
DiH_2O	0.193	3.78	Probable Phototoxicity (PIF), Phototoxic (MPE)	1000 – 16.3	< 0.195 mg/mL
HBSS	-0.016	1.08	Non phototoxic	1000 - 16.3	< 0.75 mg/mL
	0.030	ND			
	0.071	ND			
DMSO	0.296	3.79	Probable Phototoxicity (PIF), Phototoxic (MPE)	30.0 - 0.490	10 mg/mL
	0.272	4.66			
ETOH	0.205	4.97	Probable Phototoxicity (PIF), Phototoxic (MPE)	30.0 – 0.490	5 mg/mL
	0.273	5.14	Phototoxic		
Acetone	0.405	18.1	Phototoxic	100 – 0.280	1 mg/mL
	0.293	7.48			

Table 3. Phototoxicity results (MPE and PIF values) from the definitive assays for amiodarone dissolved in various solvents and Pre-Testing Solubility Assessment Result. Dilutions of the OECD reference chemical, amiodarone, were prepared in various solvents and treated on two 96-well plates. One plate was exposed to UVA and the other was kept in the dark. The optical density values were entered into the Phototox 2.0 software to obtain the PIF and MPE values. ND = Not determined. Pre-testing solubility assessment results for each solvent.

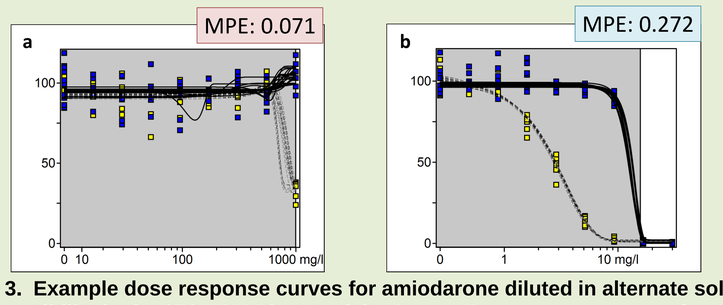

Figure 3. Example dose response curves for amiodarone diluted in alternate solvents for compatibility in the 3T3 NRU Phototoxicity Assay. Dose response curves for amiodarone prepared in HBSS (a) and DMSO (b).

DISCUSSION & FUTURE CONSIDERATIONS

- Screening of new solvents is necessary to evaluate how they affect the assay system. During the initial screening of solvents, one of the solvents (THF) resulted in probable phototoxicity (based MPE). If used as a solvent to prepare a test material, THF could enhance phototoxicity potential of the test material. Mineral oil was difficult to use in the 96-well format (due to its viscosity); and showed no interaction with the cells (lack of bioavailability) and variability in responses, all of which demonstrated its incompatibility with the test system.
- Further testing with the solvents to prepare chlorpromazine yielded promising results as the chlorpromazine fell within two standard deviations from the historical mean established at IIVS for all solvents except for HBSS. These results supported that the intermediate solvents (PEG, butylene glycol, ethanol and acetone) performed similarly to the assay positive control, chlorpromazine diluted in DMSO , and did not enhance or quench the phototoxic potential. These results support the use of these solvents, in addition to those mentioned in OECD TG 432, providing additional options for dissolving particularly insoluble materials.
- Solvent selection plays a crucial role in assessment of the phototoxic potential of test materials. In the case of Amiodarone, a known phototoxic material, use of intermediate solvents was critical in the appropriate determination of phototoxicity potential. Amiodarone was consistently predicted to have phototoxic potential when prepared in the intermediate solvents, but was incorrectly predicted when directly diluted in the primary solvent, HBSS. The PIF and MPE results for the Amiodarone dissolved in the intermediate solvents were consistent with those described in OECD TG 432 (PIF >3.25 and MPE = 0.27-0.54).

[1]*OECD Test Guideline (432) for the In Vitro 3T3 NRU Phototoxicity Test, Organization of Economic Cooperation and Development. Adopted 13 April 2004*
[2]*Anon. INVITTOX Protocol 78. 3T3 Neutral Red Uptake (NRU) Phototoxicity Assay. ECVAM DB-ALM; 2008. http://ecvam-dbalm.jrc.ec.europa.eu/*