Approaches to Evaluation of Solvents for use in Photosafety Testing

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Introduction

New Approach Methodologies (NAMs) are routinely used in photosafety testing to evaluate if a test compound has the potential to become more toxic upon exposure and subsequent exposure to light. Three such NAMs to address photosafety are the *in chemico* UV-Vis Assay, the cell-based 3T3 Neutral Red Uptake (NRU) Phototoxicity Test (PT), and the tissue-based Reconstructed human EpiDermis (RhE) Phototoxicity Test (PT), described under OECD Test Guidelines (TG) 101, 432, and 498, respectively. These dilution-based assays evaluate the test compound at multiple concentrations in solvents specified in the TGs. Additional solvents may be considered and must be thoughtfully evaluated prior to use. Evaluation of prospective solvents for photosafety testing is more nuanced because the solvents must be qualified to ensure that the novel solvent does not interfere with the assay (*i.e.*, induce phototoxicity, scavenge free radicals, quench phototoxic effect, etc.) and to demonstrate the solvent does not affect the prediction of a phototoxic reference compound. To this end, five solvents (tetrahydrofuran (THF), hexane, dimethyl sulfoxide (DMSO), ethanol, and acetone) were evaluated to understand impacts on the test systems (*e.g.*, cytotoxicity and phototoxicity potential) and their impact on identification of a known photoirritant, chlorpromazine hydrochloride (CPZ). The solvents were chosen for their known utilities in dissolving certain chemical types, as well as accessibility and cost.

Materials and Methods

Each solvent and chlorpromazine in each respective solvent were evaluated in the UV-Vis, 3T3 NRU PT, and RhE PT, as adapted from OECD TG 101, TG 432, and TG 498, respectively. A brief summary of the methodologies and prediction models for each assay are outlined in **Figures 1-3** below. In the UV-Vis assay, each solvent and CPZ in each solvent was assessed for significant absorption from 290 to 800 nm (**Figure 1**). Each solvent was analyzed (**Figure 4a**) and CPZ was diluted in each solvent at 0.003 M (**Figure 4b**) and the peak wavelength, absorbance (OD) values and corresponding Molar Extinction Coefficient (MEC) were determined. In the 3T3 NRU PT, solvents were prepared in Hanks' Balanced Salt Solution (HBSS), and CPZ was prepared (at least 8 concentrations) in each solvent and then transferred to aqueous buffer solution prior to addition to the 3T3 cells (**Figure 2**). The dose responses are presented in **Figure 5a-e**. The viability at each concentration was used to determine the IC_{50} values, Photo Irritancy Factor (PIF), and Mean Photo Effect (MPE) to determine cytotoxicity and phototoxicity potential. In the RhE PT, the solvents were topically applied (neat) to the RhE tissues (**Figure 3**) and the Optical Density (OD) values were determined (**Figure 6a**). CPZ was diluted in each solvent at 0.02% and the OD values (**Figure 6b**) were used to calculate the % relative viability and the difference in viability between irradiated (+Irr) and non-irradiated (-Irr) exposed groups. A summary of the data are presented in **Table 1**.

UV-VIS Absorption Spectra Determination (OECD TG 101)

Goal: To determine if a compound significantly absorbs light in the UV or visible light range (290-700 nm)
Applications: If a compound absorbs light, there is potential for compound to undergo photochemical degradation; further testing required
Solvents specified in the TG: Aqueous media or a suitable organic solvent such as methanol

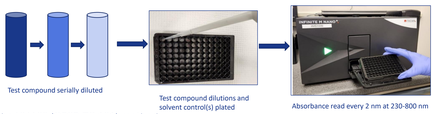

Figure 1: General outline of the UV-VIS (OECD TG 101) method

Interpreting Results for OECD TG 101

Prediction	Molar Extinction Coefficient (MEC)
Significant Absorption (290-700 nm)	MEC ≥ 1000 L mol-1 cm-1
Absorption not Significant (290- 700 nm)	MEC < 1000 L mol-1 cm-1

The absorption of the solvent should not vary more than ± 0.05 from the nominal zero value.

3T3 Neutral Red Uptake Phototoxicity Test (OECD TG 432)

Purpose: To identify if a test compound has phototoxicity potential (hazard)
Applications: Ideal screening assay; high sensitivity; high negative predictivity; if a compound has phototoxicity hazard, additional testing may be needed to address hazard
Solvents specified in the TG: Buffered salt solution, or DMSO and Ethanol (dissolved in aqueous vehicle)

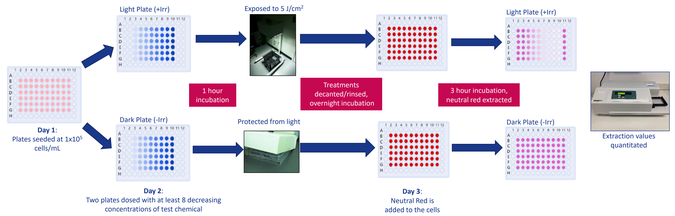

Figure 2: General outline of the 3T3 NRU Phototoxicity (OECD TG 432) method

Interpreting Results for OECD TG 432

Prediction	PIF		MPE
No Phototoxicity	PIF < 2	or	MPE < 0.1
Equivocal Phototoxicity	PIF ≥ 2 and < 5	or	MPE ≥ 0.1 and < 0.15
Phototoxicity	PIF ≥ 5	or	MPE ≥ 0.15

Per OECD TG 432: equivocal, borderline, or unclear results should be clarified by further testing.

Solvents used must be non-cytotoxic at tested concentrations (*i.e.*, concentration of the test compound); Solvent comparison >80% OD +Irr/ OD -Irr

Reconstructed Human EpiDermis (RhE) Phototoxicity Test (OECD TG 498)

Purpose: To identify if a test compound has phototoxicity potential (hazard); may also be used to address risk
Applications: Not limited by solubility; tiered testing with 3T3 NRU PT; model mimics outermost layer of skin epidermis; cells of human origin with functional *stratum corneum*; may be modified to model systemic exposures
Solvents specified in the TG: buffered salt solutions (like DPBS or HBSS), sesame seed oil, acetone in olive oil, or mineral oil.

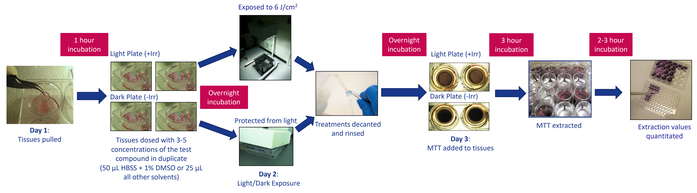

Figure 3: General outline of the RhE Phototoxicity (OECD TG 498) method

Interpreting Results for OECD TG 498

Prediction	Viability +Irr – Viability -Irr
No Phototoxicity	<30% at any concentration (up to 10%)
Equivocal Phototoxicity	30±5% at only 1 concentration
Phototoxicity	≥30% in at least one concentration

Per OECD TG 498: equivocal, borderline, or unclear results should be clarified by further testing.

The solvent control OD values (+/- Irr) should be between 0.8 and 2.8.

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References:
Organization for Economic Cooperation and Development (OECD) Test Guideline (101) "UV-VIS Absorption Spectra"(Spectrophotometric Method) (1981)
Organization for Economic Cooperation and Development (OECD) Test Guideline (432) for the In Vitro 3T3 NRU Phototoxicity Test (2019)
Organization for Economic Cooperation and Development (OECD) Test Guideline (498): In vitro Phototoxicity - Reconstructed Human Epidermis Phototoxicity test method (2023)

Results

	UV-Vis (OECD TG 101)						3T3 Phototoxicity (OECD TG 432)							RhE Phototoxicity (OECD TG 498)					
	Solubility	Conc. (M)	Peak Wavelength (nm)	Peak OD value*	Peak MEC value*	Absorption?	Solubility	Conc. (µg/mL)	IC_{50} (+Irr) (µg/mL)	IC_{50} (-Irr) (µg/mL)	PIF Value	MPE Value	Phototoxic?	Solubility	Conc. (%)	OD value (+Irr)*	OD value (-Irr)*	% Viability difference	Phototoxic?
THF	NA	NA	290	0.675	NA	Yes	Soluble	3725-100000	78589	19308	2.46	0.138	Probable	NA	NA	0.162	0.177	NA	No - Cytotoxic
Hexane	NA	NA	290	0.064	NA	No	Insoluble	1633-100000	47107	>100000^	ND	-0.014	No	NA	NA	2.04	2.08	NA	No
DMSO	NA	NA	290	0.294	NA	Yes	Soluble	3725-100000	>100000^	80112	ND	-0.034	No	NA	NA	1.67	1.72	NA	No
Ethanol	NA	NA	290	0.057	NA	No	Soluble	1633-100000	45631	51659	1.13	0.060	No	NA	NA	1.27	1.35	NA	No
Acetone	NA	NA	~230-308	>4.0[!]	NA	Yes	Soluble	1633-100000	47788	70166	1.55	0.046	No	NA	NA	1.80	1.82	NA	No
CPZ in THF	Soluble	0.003	308	3.64	4271	Yes	Insoluble	0.156-100	0.700	15.2	21.7	0.544	Yes	Soluble	0.02%	0.158	0.151	17.6%	No - Cytotoxic
CPZ in Hexane	Insoluble	0.003	290	0.014	16.8	No	Insoluble	0.156-100	>9.53^	52.0	ND	0.278	Yes	Insoluble	0.02%	1.99	2.06	1.40%	No
CPZ in DMSO	Soluble	0.003	308	3.09	3625	Yes	Soluble	0.156-100	0.848	18.0	21.3	0.450	Yes	Soluble	0.02%	1.03	1.81	43.2%	Yes
CPZ in Ethanol	Soluble	0.003	308	2.60	3049	Yes	Soluble	0.156-100	1.05	29.3	28.1	0.553	Yes	Soluble	0.02%	0.179	1.37	96.8%	Yes
CPZ in Acetone	Insoluble	0.003	316+	3.32+	3896+	Inconclusive+	Soluble	0.156-100	0.908	24.9	27.4	0.700	Yes	Soluble	0.02%	0.313	1.54	67.5%	Yes

NA – Not Applicable; ND – Not Determined; [!] - The spectrophotometer maximum absorbance was 4.0; * - Value is the average of two replicates; + - One replicate produced the above results, however this peak was not present in the second replicate and therefore inconclusive; Cytotoxicity- insufficient viability to analyze
^ - An IC_{50} (µg/mL) value could not be determined; therefore, the IC_{50} value was presented as greater than the highest dose tested

Table 1: Summary of Results for solubility, concentrations tested (if applicable), and relevant data for each solvent and CPZ dilution in each assay. Prediction models for each assay can be found in Materials and Methods.

UV-Vis Results

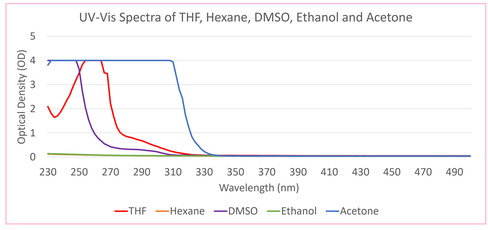


Figure 4a

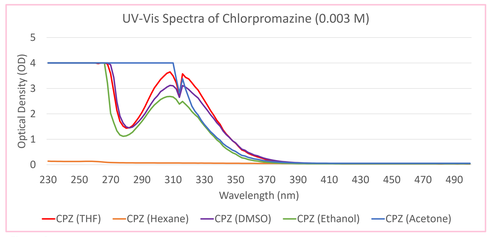
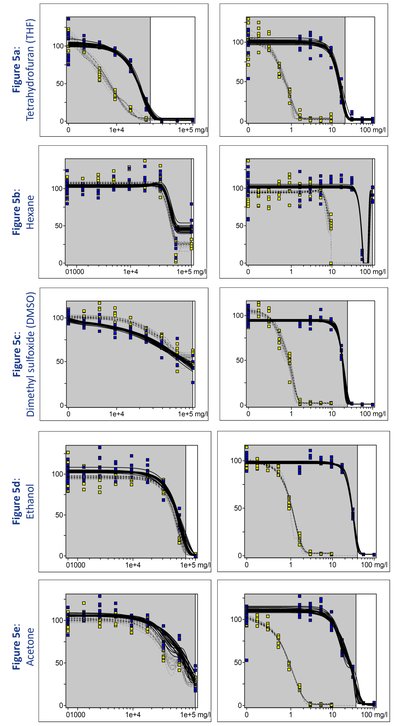


Figure 4b

Figure 4a-b. Absorption spectra of THF (red line), hexane (orange line), DMSO (purple line), ethanol (green line), and acetone (blue line) from 230 to 490 nm. Absorption (presented as optical density, OD) of each solvent (4a) and CPZ at 0.003 M in each solvent (4b).

3T3 NRU PT



RhE PT

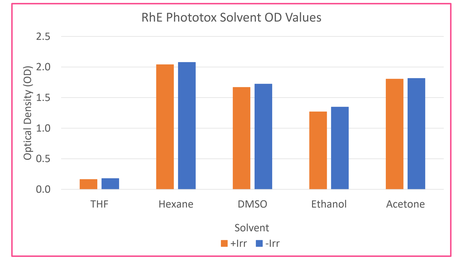


Figure 6a

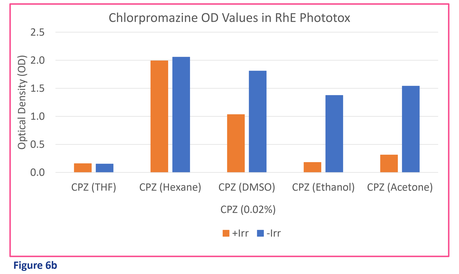


Figure 6b

Figure 6a-b. Comparison of the OD values of each solvent in the presence (orange bar) and absence (blue bar) of irradiation. The OD values of tissues exposed to each solvent alone (6a) and of tissues exposed to 0.02% CPZ prepared in each solvent (6b).

Figure 5a-e. 3T3 cell dose responses in the presence (yellow boxes) and absence (blue boxes) of irradiation as % relative viability (y-axis) over concentration (x-axis). Each solvent was prepared in aqueous diluent (HBSS) at multiple concentrations (left panels) and then each solvent was used to prepare CPZ with dose responses (right panel). Graphics generated from Phototox 2.0 software (OECD).

Conclusions

Solubility plays a critical role in dilution based assays, and solvent selection can impact the overall result. Additional considerations in solvent selection are required for photosafety assays due to potential for solvents to become photoreactive, with the incorporation of additional exposure conditions utilizing irradiation, and in an understanding with the assay requirements of sufficient viability in comparing responses in the presence and absence of irradiation. To this end, we evaluated five prospective solvents in three photosafety NAMs to understand their compatibility with the test systems. Our work demonstrates that when full solubility cannot be achieved, photoirritancy potential can still be predicted. In considering prospective solvents, preliminary evaluations must be done to confirm compatibility with each test system to ensure the solvent does not enhance or diminish effect, and subsequently, to ensure test method ability to appropriately predict phototoxicity potential.

Our findings support that the five solvents evaluated may not be fully compatible with all test systems, but have demonstrated utility within individual test systems. THF showed significant absorption in the UV-Vis assay, produced probable phototoxicity in the 3T3 NRU PT, and resulted in cytotoxicity in the RhE PT method, and therefore is not a suitable solvent for these test systems. Hexane, a non-polar solvent, is promising for UV-Vis for its ability to solubilize more complex test compounds, but was not a suitable for solubilization of CPZ in the 3T3 NRU PT (variable responses) and RhE PT (not correctly predicted at 0.02%). Although DMSO and acetone showed some absorption in the UV-Vis assay, they were suitable for the 3T3 NRU PT and RhE PT assays. Ethanol did not have significant absorption in the UV-Vis assay, was suitable for the 3T3 NRU PT and RhE PT (but resulted in lower OD values).