MB Research Labs
Experience and Innovation

TOXNOTE

3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT)

in vitro phototoxicity screening test

Phototoxicity - A toxic response froma substance applied to the skin which is either elicited or increased after subsequent exposure to light, or is induced by skin irradiation after system administration of a chemical.

The cytotoxicity and phototoxicity of test compounds in 3T3 cells (in the presence or absence of solar simulated light) is assessed by Neutral Red Uptake.

MB Research Labs offers the 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT) as a standard protocol which is conducted by our team of scientists under Good Laboratory Practices.

The MB Research 3T3 NRU PT protocol is based on OECD test guidelines and is designed to detect the phototoxicity induced by the combined action of a test article and light by using an in vitro cytotoxicity assay with the Balb/c 3T3 mouse fibroblast cell line. The test identifies aqueous-soluble compounds (or formulations) that have the potential to exhibit in vivo phototoxicity after systemic application.

For more information about phototoxicity testing see: WWW.3T3NRU.COM

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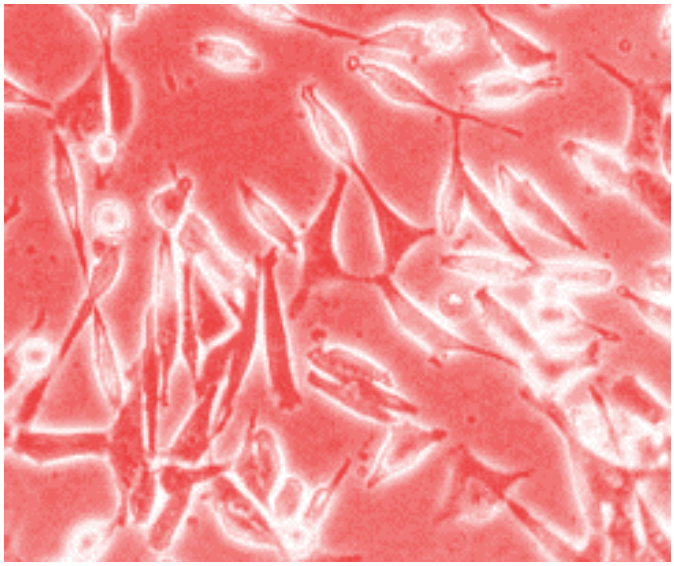
***in vitro* phototoxicity screening test**

BACKGROUND:

The 3T3 Neutral Red Uptake Phototoxicity Assay (3T3 NRU PT) can be utilized to identify the phototoxic effect of a test substance induced by the combination of test substance and light. The 3T3 NRU PT assesses the cytotoxic effect of a test substance after exposure to a non-cytotoxic dose of UVA/VIS light compared to the absence of exposure. Cytotoxicity is expressed as a concentration-dependent reduction of the uptake of the vital dye - Neutral Red.



Balb/c 3T3 fibroblasts.

Substances that are phototoxic *in vivo* after systemic application and distribution to the skin, as well as compounds that could act as phototoxicants after topical application to the skin can be identified by the test. The reliability and relevance of the 3T3 NRU PT have been evaluated and has been shown to be predictive when compared with acute phototoxicity effects *in vivo* in animals and humans.

REGULATORY BACKGROUND:

The 3T3 NRU PT test was developed and validated in a joint EU/COLIPA project from 1992-1997.

In 1996, the 3T3 NRU PT was recommended by OECD as an *in vitro* approach for the assessment of chemicals phototoxicity potential.

In April 2004, the 3T3 NRU PT protocol was finalized and adopted as the OECD 432 protocol, In Vitro 3T3 NRU phototoxicity test.

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Experimental Design

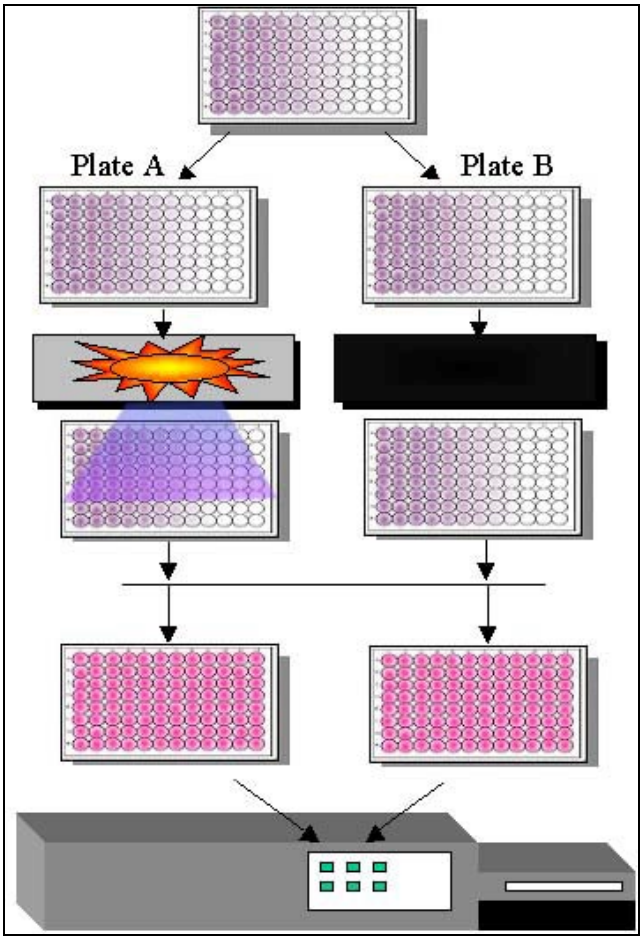
Balb/c 3T3 cells (Lot#2680275) are plated and grown to 60-80% confluence in 6 cm culture plates (37°C, 5% CO2) in growth medium (DMEM containing 1.5 g/L NaHCO3 and 10% newborn calf serum) to obtain a sufficient quantity of cells for the experiment. Cells are harvested by trypsinization, and plated at a density of 10,000 cells/well in the central 60 wells of 96-well microplates.

After allowing for cell attachment overnight, the medium is replaced with HBSS buffer containing the test article or CPZ positive control. Each dosing group consists of six (6) wells treated identically with one concentration of the test article per column. Vehicle controls are placed on the left (column 2) and right (column 11) of the dosing area and consist of buffer alone. The cells are incubated for 1 hour at 37°C and 5% CO2. Two identically treated plates (except for the concentrations of test article ±UVA) are prepared for the test article as well as for the CPZ control. Following incubation, one of each set of plates are irradiated using a Solar Simulator as per MB Research Laboratories SOPs.

Measurement of the UVA and UVB energy output of the light source is performed prior to irradiation to ensure the exposure time would accurately achieve ~5 J/cm2 UVA with less than 1% UVB. The matching dark plate (unirradiated) is kept at room temperature in a dark box for the same amount of time. After UVA irradiation, the test solution is removed from both plates of each set; the wells are washed with HBSS and re-fed culture medium.

Following test article exposure for 20-24 hours at 37°C and 5% CO2, the medium is replaced with Neutral Red Medium and the cells are then returned to the incubator for an additional 3 hours. Following the 3-hour uptake of the neutral red, the medium is discarded, the wells are washed with HBSS, and extraction buffer (1.0% Glacial Acetic Acid, 50% Ethanol, 49% dH2O) is added to each well.

Following a 30-minute extraction period with mixing, the absorbance of each well is measured at 540 nm on a MicroQuant plate reader (Bio-Tek Instruments). The mean OD values of the outer wells (blanks) are used as a reference.



Basic MB 3T3 NRU PT Procedural Outline
Seed 96 well plates with 3T3 cells
Dose 2 plates with 8 concentrations of Test Article. Incubate 37°, 5% CO_2, 1 hr.
Plate A: Expose to 5 J/cm². Plate B: Keep in the dark.
Return to 37°, 5% CO_2, 24 hrs.
Add Neutral Red media, Incubate 3 hrs. Rinse and Fix.
Read OD at 540 nm Determine IC50UVA and IC50Dark.
Calculate Photo Irritation Factor. Ratio; if > 6 then = Phototoxin.

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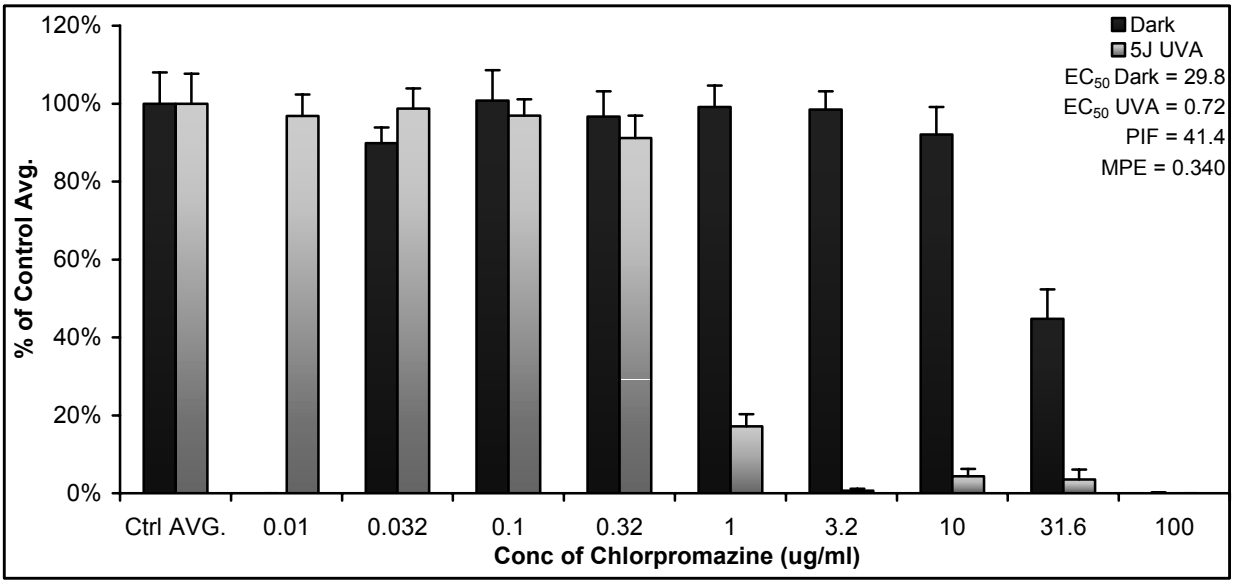
Quality Check of the Assay:



Positive Control:
For each phototoxicity assay performed, neutral red uptake of 3T3 cells following treatment with the positive control CPZ is determined in the absence (–UVA) and presence (+UVA) of irradiation (5 J/cm2). A test meets acceptance criteria if, for CPZ:

EC50 –UVA is in the range of 7.0 – 90.0 ug/ml;
EC50 +UVA is in the range of 0.1 – 2.0 ug/ml;
The Photo Irritation Factor (PIF = EC50 –UVA/ EC50 +UVA) is at least 6.0.

Negative Control:
The mean OD540 of vehicle-treated controls must be at least 0.3, which is an indication that the seeded cells are growing exponentially. The left (row 2) and right (row 11) mean vehicle-treated controls must not differ by more than 15% from the mean of all controls, which is a check for systematic errors.

Analysis of Data and Criteria for Phototoxic Potential
The mean absorbance value for each dose group was calculated and expressed as percent viability for each sample using the following formula:

% viability = 100 X (OD sample/ OD negative control)

The percent viability was plotted on the y-axis (linear) versus the dose (log x-axis).

Phototoxicity determination is based on the calculation of the **Photo-Irritant Factor (PIF)**. The PIF model compares the effect of a compound in the presence (+UVA) and absence (-UVA) of UVA irradiation at a single concentration — that concentration at which cytotoxicity occurs for 50% of the cells. ***According to this model, a compound is considered to have phototoxic potential if PIF = EC50 –UVA/ EC50 +UVA > 5.0. The PIF model predicts "probable phototoxicity" if the PIF >2.0 and <5.0.***

If the EC50 in the presence or absence of light cannot be calculated, phototoxicity is determined by the Mean Photo Effect (MPE) using the 3T3 NRU Phototoxicity Prediction Software developed by ZEBET. This software uses the algorithms published by Holzhutter (1997) and predicts the phototoxic potential of a test substance based on comparison of the two complete concentration-response curves concurrently obtained in the presence (+UVA) and absence (–UVA) of UVA irradiation. According to the MPE model, a test substance is considered to have phototoxic potential if the MPE> 0.150. Also, a test substance is considered to exhibit "probable phototoxicity" if the MPE >0.100 and <0.150.

3T3 NRU PT VALIDATION STUDIES

Objective:

To evaluate the phototoxicicity induced by the combined action of a chemical and light by using an in vitro cytotoxicity assay with the Balb/c 3T3 mouse fibroblast cell line.

Method Synopsis:

A slightly modified version of the protocol outlined in the OECD 432 guideline was followed for the validation of the 3T3 NRU Phototoxicity Assay. A Honle SOL 500 solar simulator was used with an H1 filter as the UV light source. Balb/c 3T3 cells were seeded in the central 60 wells of two 96-well plates and maintained in culture for 24 hours. The duplicate 96-well plates were then preincubated with eight different concentrations of the test article for 1 hour. Then one plate was exposed to a dose of 5 J/cm2 UVA while the other plate was kept in the dark. After UV irradiation, the treatment medium was replaced with culture medium and, after 24 hours, cell viability was determined by neutral red uptake for 3 hours.

Summary:

The 3T3 NRU Phototoxicity Assay, an in vitro assessment of the phototoxic potential of a substance was validated following OECD 432 Guidelines. The validation of this assay involved testing eight chemicals with various phototoxic potential. The results of the definitive test were in accordance with the results obtained by the OECD, which were published in the 432 Guideline. Protoporphyrin IX, chlorpromazine (CPZ), anthracene, amiodarone and norfloxacin were classified correctly as phototoxins while hexachlorophene, sodium lauryl sulfate (SLS) and L-histidine were not phototoxic. These results demonstrate the validity of the adapted 3T3 NRU Phototoxicity assay used at MB Research.

3T3 NRU Phototoxicity Assay Validation Definitive Test (n=6)

Positive Phototoxin	EC_{50} Dark (ug/ml)	EC_{50} 5J UVA (ug/ml)	PIF	MPE	Phototoxicity	
					PIF	MPE
Protoporphyrin IX	>316	0.44	>719	0.805	+	+
Chlorpromazine (expt 1)	29.8	0.72	41.4	0.340	+	+
(expt 2)	56.1	1.47	38.4	0.278	+	+
(expt 3)	24.4	0.91	27.0	0.326	+	+
Anthracene	>31.6	2.5	>12.5	0.315	+	+
Amiodarone	59.0	6.3	9.4	0.216	+	+
Norfloxacin	>316	54.1	>5.9	0.179	+	+
Negative Phototoxin						
Hexachlorophene	3.10	2.80	1.1	0.023	–	–
Sodium Lauryl Sulfate	17.5	21.4	0.8	0.027	–	–
L-Histidine	>5000	>5000	No PIF	-0.015	–	–

A PIF Value >5 and MPE Value > 0.150 is predictive of a phototoxin.

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Contingency Table.

	Positive Phototoxin	Negative Phototoxin	Total
Tested +	**7**	**0**	**7**
Tested -	**0**	**3**	**3**
Total	**7**	**3**	**10**

Accuracy =	**10/10**	**100%**
Sensitivity =	**7/7**	**100%**
Specificity =	**3/3**	**100%**

Positive Predictivity =	**7/7**	**100%**
Negative Predictivity =	**3/3**	**100%**

Test Articles

Chemical	Source	Lot #	Solvent	Description
Amiodarone Hydrochloride	Sigma A8423	061K1087	DMSO	White powder
Anthracene	Sigma Aldrich 141062	13721HC	DMSO	White powder
Chlorpromazine	Sigma C-0982	018H11721	DMSO/ Ethanol	White powder
Hexachlorophene	Aldrich 30,711-4 [70-30-4]	KX 08414JW	DMSO	Brown powder
L-Histidine	Fluka Biochemika 53320	1100344 62904043	Ethanol	White powder
Norfloxacin	Sigma N9890	083H0921	DMSO	White powder
Protoporphyrin IX, Disodium Salt	Aldrich 258385	04505BC	Ethanol	Dark purple powder
Sodium lauryl sulfate	Sigma L4509	043K0059	Ethanol	White powder

Results and Discussion

Solubility:
Due to the limited solubility of the majority of the test chemicals in aqueous buffer(s), non-toxic organic (as suggested by OECD 432) solvents were used. DMSO was used for anthracene, amiodarone, norfloxacin and hexachlorophene. Ethanol was used for protoporphyrin IX, SLS and L-Histidine. Both solvents were tested side-by-side with chlorpromazine depending on which test substances were being tested and their solubilities. The test chemicals were prepared in the solvent at 1000 times the highest dosing concentration. The 1000X solutions were then diluted by adding 10ul of the solution to 9.990 ml of HBSS. The resulting solution was 0.1% solvent in the highest dosing concentration of the test chemical. The remaining concentrations were prepared by diluting the highest concentration with HBSS containing 0.1% solvent.

Screening Test:
A screen was performed to assure that: 1) the appropriate concentration ranges were chosen and 2) to check the proficiency of the test system. During the screen, six chemicals matched results from OECD 432 guidelines using both the PIF and MPE analysis methods. Two chemicals demonstrated conflicting results and were retested during the screen assay. Both chemicals (Hexachlorophene and Sodium Lauryl Sulfate) were correctly classified as negative according to MPE analysis the second time they were tested.

Definitive Test:
A definitive test was performed on the seven test chemicals using chlorpromazine as the positive control. All of the test chemicals were classified correctly according to the OECD 432 guidelines. All test chemicals were classified correctly as either a phototoxin or non-phototoxin. Several of the test chemicals showed no cytotoxic effects at the highest concentrations making it difficult to compare the PIF values obtained at MB with those from the OECD guideline.

Conclusion

Based on the results obtained from the definitive test, the adapted 3T3 NRU PT assay will accurately predict the phototoxic potential of test articles that are soluble in the buffering solutions used as the vehicles for this assay.

References:
OECD. OECD Guideline for Testing of Chemicals: No. 432 -- In Vitro 3T3 NRU Phototoxicity Test. Anonymous. Anonymous. 2004.

Anonymous. Manual for 3T3 NRU Phototox Software, Version 2.0. BfR Zebet, 2002.

H. G. Holzhutter. A general measure of in vitro phototoxicity derived from pairs of dose-response curves and its use for predicting the in vivo phototoxicity of chemicals. ATLA 25:445-462, 1997. (Abstract)

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