

NEUTRAL RED UPTAKE PHOTOTOXICITY ASSAY IN BALB/c 3T3 MOUSE FIBROBLASTS

Theory: A decrease in the uptake of neutral red dye (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) by Balb/c 3T3 mouse fibroblast cell cultures when exposed with UVA light and without UVA light is used as an indication of the phototoxic potential of the test chemical.

Experimental Procedure

Target Cell Preparation

- Stock cell cultures are maintained at $37 \pm 1^\circ\text{C}$ in a humidified atmosphere containing $5 \pm 1\%$ CO_2 .
- Cells are subcultured when the stock culture is 50 to 80% confluent.
- A cell suspension is prepared to yield 1.0×10^5 cells/ml.
- 100 μl (~10,000 cells per well) of the cell suspension is added into the designated wells of the 96-well bioassay plate.
- The cultures are incubated for approximately 24 hours.
- Since UV sensitivity of the cells increases with aging, cells are used at passage numbers < 100. Stock cells are cultured in the absence of antibiotics and new stock cultures are initiated from the frozen working bank every 3 months.

Sample and Positive Control Preparation

- On the day of dosing, the test chemical is suspended in EBSS/HBSS (2X concentration) if workable, or another appropriate solvent (DMSO or ethanol, with a final concentration of 1% or 0.5%, respectively).
- A total of eight dilutions of the test chemical are made for the dose range finding assay (100 $\mu\text{g/ml}$ to 0.03 $\mu\text{g/ml}$ in $\frac{1}{2}$ log increments) and a total of eight dilutions are made for the definitive assays based on the results of the dose range finding assay.
- Chlorpromazine is used as the positive control. Eight concentrations (100 $\mu\text{g/ml}$ to 1.77 $\mu\text{g/ml}$ for the plate not exposed to UVA light (dark) and 9.96 $\mu\text{g/ml}$ to 0.176 $\mu\text{g/ml}$ for the plate exposed to UVA light) are tested.

Assay Procedure

- 24 hours after seeding the cells into 96-well plates, the growth media is removed, cells rinsed once with 125 μl EBSS or HBSS, and the appropriate wells were refed with 50 μl of the dosing vehicle (EBSS/HBSS). The outer wells were refed with 100 μl of isotonic solution to increase the humidity.
- Two plates were designated for each test material; one plate for determination of cytotoxicity (dark) and the second plate for determination of phototoxicity (UVA). 50 μl of the test and control article dilutions are added to the appropriate wells yielding. The cells are exposed to the test article dilutions for approximately 1 hour.
- After the one hour treatment, one plate is exposed to 1.7 ± 0.1 mW/cm² of UVA light and the second plate was placed in the dark at room temperature, both incubated for 50 ± 2 minutes. The plates are then rinsed at least once and refed with 100 μl of Assay Medium (growth medium supplemented with pen/strep).
- Prior to the neutral red addition, the wells are evaluated microscopically for cytotoxicity.
- Approximately 24 hours after the post-treatment incubation, the assay media is removed from the wells and replaced with 100 μl of neutral red solution (50 $\mu\text{g/ml}$ neutral red in assay medium). 100 μl of isotonic solution is added to the blank wells (outer wells).
- The 96-well plates are returned to the incubator for 3 hours to allow the neutral red uptake within the viable cells.
- After 3 hours, the neutral red solution is removed and rinsed once with 250 μl EBSS or HBSS. Next, 100 μl of solvent (EtOH and Acetic Acid) are added.
- The neutral red is extracted from the cultures for at least 20 minutes at room temperature while shaking.
- The absorbance of the neutral red at 550 nm (OD_{550}) is measured with a 96-well plate reader.

Data Evaluation

- The relative survival of each treatment group is determined by comparing the mean corrected OD_{550} of the test article-treated wells to the mean corrected OD_{550} of the solvent control wells.
- Dose response curves may be plotted with the % of control on the ordinate and the test article doses on the abscissa.
- The IC_{50} (the concentration of the test article that inhibits the uptake of neutral red by 50%) is determined by interpolation from the dose response curves.
- A Photo-Irritancy Factor (PIF) value and a Mean Photo Effect (MPE) Value were calculated for each test article using the software program NRU-PIT2. The PIF is determined by comparing the IC_{50} without UVA (dark) with the IC_{50} with UVA to determine the "factor" difference.

$$\text{FACTOR} = \text{IC}_{50} (-\text{UVA}) / \text{IC}_{50} (+\text{UVA})$$
 A factor >5 is indicative of phototoxic potential of the test material.
- The MPE measures the effect of UV exposure over a range of concentrations. A material is considered nonphototoxic if the MPE is < 0.1 and phototoxic if the MPE is ≥ 0.1 .