Qualification of Cell Line A549 for the Neutral Red Uptake (NRU) Assay

Shannon Bruce[1], Khushbu Garala[1], Deepa Srinivasan[1], Sandra Springer[1], Douglass Dey[1], Kayla Campasino[1], Rohan Kulkarni[1]
[1]MilliporeSigma BioReliance® Toxicology Testing Services, Rockville, Maryland

Introduction

As per 3R, in vitro cytotoxicity tests such as the Neutral Red Update (NRU) assay are routinely used as alternative toxicity tests to eliminate the use of animals for acute oral toxicity tests. The NRU assay is one of the most commonly used cytotoxicity assays for chemicals, pharmaceuticals, cigarette smoke condensate, plant extracts and medical device extracts. As per the ICCVAM protocol, this test is generally performed in BALB/c 3T3 cells. The modal number of chromosomes in BALB/c 3T3 cells is 78 with a range of 62 to 109. The stem line number is hypo-tetraploid which makes this a karyotypically unstable cell line. We are proposing the use of the A549 cell line which is a hypotriploid human lung adenocarcinoma epithelial cell line. The modal number of chromosomes in A549 cells is 66 with a range of 64 to 67 and can be obtained from American Type Culture Collection (ATCC). Also, as per some of the peer-reviewed literature, this lung cell line may be useful to understand the role of alveolar Type II cells in the drug or chemical delivery at the pulmonary epithelium which makes this work relevant to tobacco research. Since the ICCVAM protocol is designed for the BALB/c 3T3 cells, we have performed a comparative study using A549 cells. We tested nine chemicals, from the NTP database with known different modes of action, using the 96-well plate method. The IC20, IC50, and IC80 values were calculated for each chemical, where possible. Our results indicate that the A549 cells can be used in lieu of the BALB/c 3T3 cells for the Neutral Red Uptake Assay.

Materials

Reagents dimethyl sulfoxide (DMSO), water, sodium lauryl sulfate (SLS), penicillin/streptomycin, L-glutamine, neutral red (NR), phosphate buffered saline, and glacial acetic acid were purchased from Sigma Aldrich. Chemicals caffeine, sodium chloride, acetaminophen, aminopterin, cycloheximide, hexachlorophene, nicotine, paraquat, and sodium oxalate were purchased from MilliporeSigma. Kaighn's Modification of Ham's F12 medium (F-12K) and Dulbecco's Modified Eagle's medium (DMEM) was purchased from ThermoScientific. Fetal bovine serum (not heat-inactivated) and newborn calf serum (heat-inactivated) was purchased from GE Healthcare LifeSciences.

Method

The science of this assay is based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR is a weak cationic dye that readily diffuses through the plasma membrane and concentrates in lysosomes where it electrostatically binds to the anionic lysosomal matrix. Cytotoxic test substances can alter the cell membrane and/or the lysosomal membrane to cause irreversible adverse changes. Dead cells with damaged membrane(s) have limited ability to uptake the neutral red dye. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of NR following exposure to test substance. A spectrometer is used to measure the absorbance of each sample at a specific wavelength. The absorbance values (optical density) are then used to determine cell viability by comparing the optical density (OD) of each well treated with test material to the negative (or vehicle) control wells.

Procedure

Cells were seeded at an appropriate density for the cell line in a 100 µL aliquot. Cells were incubated overnight in a 37ºC humidified incubator with 5% CO_2.

Cell Line	Source Information	Seeding Density
BALB/c 3T3	Mus musculus embryo fibroblast (ATTC CCL-163)	3 x 10^3 cells/well (100 µL aliquot)
A549	Human lung adenocarcinoma epithelial (ATCC CCL-185)	7 x 10^3 cells/well (100 µL aliquot)

After 24 ± 2 hours of incubation, the media was removed and replaced with fresh medium containing a vehicle control, chemical or positive control (SLS).

Chemical	Starting Dose Level and Spacing
Caffeine	1.7 dilutions from 4 mg/mL
Sodium chloride	1.25 dilutions from 10 mg/mL
Acetaminophen	Half dilutions from 4 mg/mL
Aminopterin	1.9 dilutions from 500 ng/mL
Cycloheximide	Half dilutions from 2.0 µg/mL
Hexachlorophene	1.9 dilutions from 25 µg/mL
Nicotine	1.4 dilutions from 3.0 mg/mL
Paraquat	Half dilutions from 2.0 mg/mL*
Sodium Oxalate	Half dilutions from 2.0 mg/mL
Sodium Lauryl Sulfate	1.4 dilutions from 100 µg/mL (BALB/c 3T3)
	1.4 dilutions from 150 µg/mL (A549)

* Additional testing for A549 cell line using half dilutions from 100 µg/mL.

After addition of fresh medium, cells were incubated for 48 ± 0.5 hours in a 37ºC humidified incubator with 5% CO_2. Cells are examined for signs of toxicity or precipitate at the end of the treatment period.

Following treatment with vehicle or positive control, media was removed and replaced with medium containing NR dye (25 µg/mL). Cells were incubated with NR dye for approximately 3 hours in a 37ºC humidified incubator with 5% CO_2. Cells are examined to determine the presence of NR crystals within the cellular structure. Cells were washed once with PBS and desorb solution was added to extract the NR dye. A spectrophotometer was used to measure absorption at 540 nm.

Results

The IC20, IC50, and IC80 values were calculated for each chemical, where possible. For the BALB/c 3T3 cell line, values were determined for 7 of 9 chemicals. For the A549 cell line, values were determined for 7 of 9 chemicals.

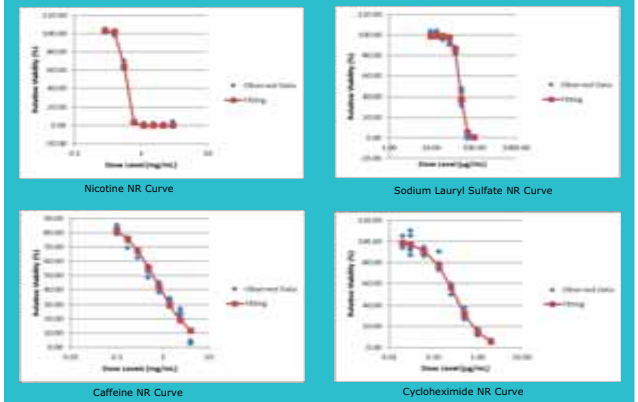
Chemical	BALB/c 3T3 Cell Results		
	IC_{20} value	IC_{50} value	IC_{80} value
Caffeine	0.17 mg/mL	0.58 mg/mL	1.97 mg/mL
Sodium chloride	6.87 mg/mL	8.96 mg/mL	11.68 mg/mL
Acetaminophen	ND	ND	ND
Aminopterin	0.00 ng/mL	9.17 ng/mL	18.82 µg/mL
Cycloheximide	0.12 µg/mL	0.30 µg/mL	0.79 µg/mL
Hexachlorophene	6.54 µg/mL	11.68 µg/mL	20.85 µg/mL
Nicotine	0.53 mg/mL	0.60 mg/mL	0.68 mg/mL
Paraquat	ND	ND	ND
Sodium Oxalate	0.12 mg/mL	0.20 mg/mL	0.34 mg/mL

ND = Not determined due to viability >50% at the top concentration tested.

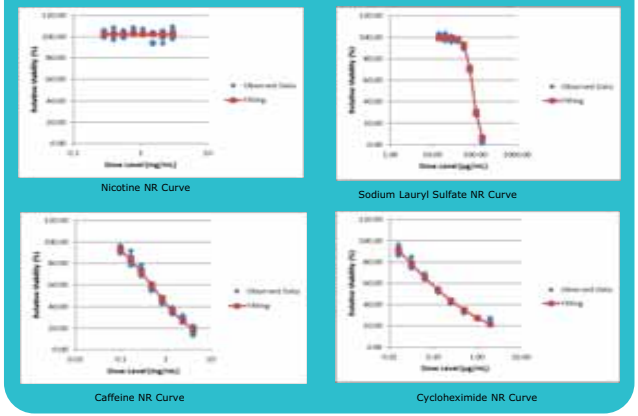
Chemical	A549 Cell Results		
	IC_{20} value	IC_{50} value	IC_{80} value
Caffeine	0.13 mg/mL	0.77 mg/mL	4.54 mg/mL
Sodium chloride	5.04 mg/mL	8.92 mg/mL	15.79 mg/mL
Acetaminophen	0.50 mg/mL	1.16 mg/mL	2.72 mg/mL
Aminopterin	ND	ND	ND
Cycloheximide	0.01 µg/mL	0.16 µg/mL	5.11 µg/mL
Hexachlorophene	2.46 µg/mL	13.35 µg/mL	72.46 µg/mL
Nicotine	ND	ND	ND
Paraquat	14.37 µg/mL	24.40 µg/mL	4.42 µg/mL
Sodium Oxalate	0.06 mg/mL	0.11 mg/mL	0.20 mg/mL

ND = Not determined due to viability >50% at the top concentration tested.

BALB/c 3T3 Data



A549 Data



Summary

A549 cell data from the nine chemicals tested in the qualification yield similar inhibitory concentration (IC) values and analysis curves. From the data presented, the A549 cell line can be used in lieu of the BALB/c 3T3 cell line within the Neutral Red Uptake Assay.

References

Borenfreund, E. and J.A. Puerner. 1985. Toxicity determination in vitro by morphological alterations and neutral red absorption. Toxicol. Lett. 24: 119-124.

Babich, H. and Borenfreund, E. 1992. Neutral red assay for toxicology in vitro. In Vitro Methods of Toxicology (R. R. Watson, ed.)pp. 237-251. CRC Press, Boca Raton, Fla.

ICCVAM-Recommended Test Method Protocol BALB/c 3T3 NRU Cytotoxicity Test Method Originally published as Appendix C1 of "ICCVAM Test Method Evaluation Report: In Vitro Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Tests" NIH Publication No. 07-4519 – Published 2006 Available at: http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_tmer.htm

OECD Guidance Document on Using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests, No. 129 (20 Jul 2010).