A Comparison of BALB/c 3T3 and CHO-WBL Cell Lines in the Neutral Red Uptake Assay

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charles river

1 ABSTRACT

Charles River - Skokie, has established methods for the Neutral Red Uptake Assay (NRU) using BALB/c 3T3 cells in accordance with the OECD Test Guideline 129 as well as Chinese Hamster Ovary (CHO) - WBL cells in accordance with the Health Canada Test Guidance T-502. The NRU Assay is used to generate in vitro cytotoxicity data for a variety of chemicals, cigarette smoke fractions, e-liquids and medical devices.
Briefly, BALB/c 3T3 and CHO-WBL cells are exposed to a range of test article concentrations in a 96-well plate for 48 or 24 hours respectively. At the end of exposure, the test article is removed and neutral red (NR) is added to the cells for an incubation of 3-hours. Viable cells will incorporate and bind the NR, such that cytotoxicity is expressed as a concentration-dependent reduction in the uptake of NR. Following incubation, the NR media is removed, cells are washed, and a desorb media is added to extract the NR from the cells. Absorption is measured at 540 nm. For comparison; the data generated is fitted with a 4-parameter curve, and the IC50, the concentration at which 50% cytotoxicity is observed, is calculated. Absorbance fractions were also calculated as a percent of the negative control to provide the relative absorbance in accordance with the Health Canada Methods.

Wells were seeded at a density of 1x104 cells/well. BALB/c 3T3 cells were exposed for 48 hours to the positive control, sodium lauryl sulfate (SLS). To accommodate the faster doubling time (12-14 hours for CHO-WBL compared to 18-22 hours for BALB/c 3T3), the exposure period of the CHO-WBL cells was 24 hours. Both cell lines underwent approximately two doublings in the presence of SLS. Eight concentrations of SLS were examined in the BALB/c 3T3 cell line ranged from 6.8 to 100 µg/mL using a 1.21 dilution factor. To obtain a similar cytotoxicity curve as with the BALB/c 3T3 cells the SLS in the CHO-WBL NRU assay was increased to 13.6 to 200 µg/mL. In total over 10 trials in each cell line were conducted.

The concentrations tested produced a cytotoxicity fitted dose-response curve with an R^2 (coefficient of determination) of ≥ 0.85 for the Hill model fit. Based on this model the two cell lines produced significantly different IC50 values for SLS. BALB/c 3T3 cells were more sensitive to the effects of SLS with an average IC_{50} of 19.99 ± 3.35 µg/mL. CHO-WBL cells provided a more robust response with an average IC_{50} of 89.15 ± 3.38 µg/mL. The average absorbance displayed in the vehicle control wells varied by trial, but overall was approximately the same between the two cell lines, suggesting similar growth, final cell density and similar uptake of the NR dye across the two cell lines.

2 MATERIALS AND METHODS

The NRU in vitro cytotoxicity assay is based on the ability of viable cells to incorporate and bind NR, a supravital dye (Borenfreund and Puerner, 1985). 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. The concentration of NR dye desorbed from the cultured cells is directly proportional to the number of living cells. Toxic substances can alter the cell surface or the lysosomal membrane to cause lysosomal fragility and other adverse changes that gradually become irreversible. Such adverse changes cause inhibition of cell growth and/or cell death which will decrease the amount of NR retained by the culture. Cytotoxicity is expressed as a concentration-dependent reduction of the uptake of NR after chemical exposure. The NRU assay uses a 96-well plate format for the production of replicate measurements at various positive control concentrations.

One day prior to dosing, the interior wells of a 96-well flat bottom plate were seeded with either BALB/c 3T3 cells or CHO-WBL cells at a density of $1x10^4$ cells/well. The perimeter of the plate was filled with growth media only (no cells). Plates were incubated at 37 ± 1 ° C, 5 ± 1% CO_2, >80% humidity for approximately 24 hours prior to dosing. On the day of dosing, growth media was removed by inversion of the plate and either further growth media or SLS in growth media were added to the appropriate wells. For BALB/c 3T3 cells the SLS concentrations examined were at final concentrations of 6.8, 10.0, 14.7, 21.5, 31.6, 46.4, 68.1, and 100 µg/mL. The SLS concentrations tested in CHO-WBL cells were 13.6, 20.0, 29.4, 43.0, 63.2, 92.8, 136 and 200 µg/mL. After addition of the SLS, the plates were returned to the incubator for approximately 24 or 48 hours for CHO or BALB cells respectively. See Figure 1 for an example of the plate layout.

Following treatment, the media was removed from each well by inversion and cells were rinsed with Dulbecco's Phosphate Buffered Saline (PBS). Neutral Red (NR) dye was added to each well and the plates were incubated for an additional 3 ± 0.1 hours. After incubation, the NR dye was removed by inversion, cells were rinsed with PBS, and 100 µL of NR desorb solution (49 parts water/50 parts ethanol/1 part glacial acetic acid) were added to each well to extract the dye. Plates were incubated with shaking for 20-45 minutes and allowed to rest for at least 5 minute before reading the optical density (OD) at 540 nm using a microtiter plate reader. Data were fitted with a 4-parameter curve and the IC_{50} for each trial was determined. See Figure 2 for the equation used to determine the IC_{50}.

	1	2	3	4	5	6	7	8	9	10	11	12
A	VC_b	VC_b	C_{1b}	C_{2b}	C_{3b}	C_{4b}	C_{5b}	C_{6b}	C_{7b}	C_{8b}	VC_b	VC_b
B	VC_b	VC_1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC_2	VC_b
C	VC_b	VC_1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC_2	VC_b
D	VC_b	VC_1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC_2	VC_b
E	VC_b	VC_1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC_2	VC_b
F	VC_b	VC_1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC_2	VC_b
G	VC_b	VC_1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC_2	VC_b
H	VC_b	VC_b	C_{1b}	C_{2b}	C_{3b}	C_{4b}	C_{5b}	C_{6b}	C_{7b}	C_{8b}	VC_b	VC_b

Figure 1. Typical plate map for the NRU assay. VC – Vehicle control; C_1 – C_8 – Eight test article (or positive control) concentrations where C_1 is the highest concentration and C_8 is the lowest concentration. b – Blank well - These wells contain the test article/positive control or vehicle control, but no cells.

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log EC50 - \log X)\text{HillSlope}}}$$

Figure 2. Hill Model Function for determination of IC_{50}. The Hill function which is a four-parameter logistic mathematical model relating the concentration of the test article or positive control to the response (typically following a sigmoidal shape). Where Y=response (i.e., % viability), X is the test article or positive control concentration producing the response, Bottom is the minimum response (0% viability, maximum toxicity), Top is the maximum response (maximum viability), EC_{50} is the test article or positive control concentration at the response midway between Top and Bottom, and HillSlope describes the slope of the curve. When Top=100% viability and Bottom=0% viability, the EC_{50} is the equal to the IC_{50}.

3 RESULTS

PLATE Trial 26

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.044	0.043	0.046	0.045	0.044	0.044	0.044	0.043	0.043	0.043	0.044	0.044
B	0.045	0.295	0.048	0.045	0.044	0.046	0.046	0.098	0.182	0.249	0.332	0.045
C	0.045	0.313	0.045	0.044	0.047	0.045	0.046	0.096	0.179	0.248	0.320	0.050
D	0.044	0.301	0.047	0.045	0.044	0.043	0.044	0.090	0.170	0.245	0.336	0.043
E	0.044	0.299	0.045	0.043	0.043	0.042	0.044	0.086	0.180	0.236	0.325	0.042
F	0.045	0.300	0.046	0.044	0.044	0.044	0.045	0.089	0.162	0.226	0.307	0.045
G	0.043	0.329	0.045	0.042	0.042	0.042	0.045	0.092	0.164	0.239	0.336	0.042
H	0.048	0.044	0.046	0.045	0.045	0.045	0.045	0.044	0.047	0.045	0.043	0.044

Figure 3. Representative raw OD_{540} absorbance readings from a BALB/c 3T3 plate treated with SLS as laid out in Figure 1.

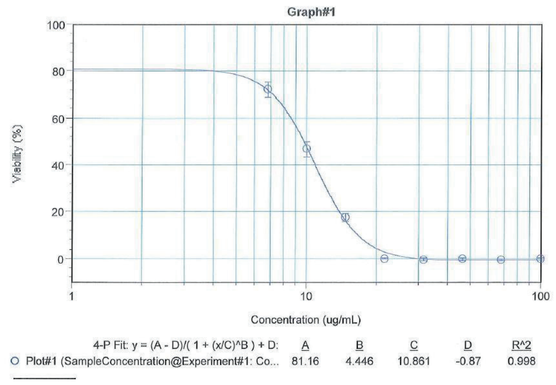

Figure 4. Representative Hill Fit curve from a SLS treated plate with BALB/c 3T3 cells. The C value is the calculated IC_{50}

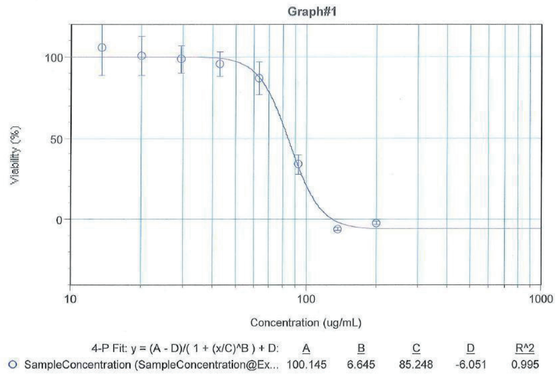

Figure 5. Representative Hill Fit curve from a SLS treated plate with CHO-WBL cells. The C value is the calculated IC_{50}

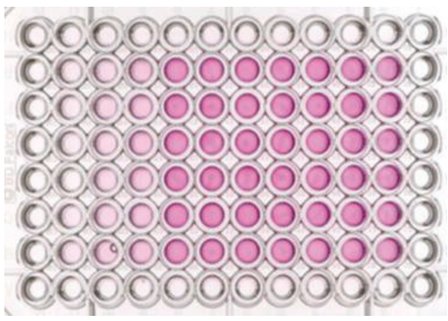

Figure 6. Neutral red uptake decreases with increasing cytotoxicity (from right to left on the plate). (Repetto et al., Nature Protocols, 2008).

	IC_{50} (µg/mL)	
	BALB/c 3T3	CHO-WBL
Mean	19.99	89.15
Standard Deviation	3.35	3.38

Figure 6. Calculated IC_{50} of SLS across a minimum of 10 trials with each cell line.

4 CONCLUSIONS

Based on this model the two cell lines produced significantly different IC_{50} values for SLS.

- BALB/c 3T3 cells were more sensitive to the effects of SLS with an average IC_{50} of 19.99 ± 3.35 µg/mL.
- CHO-WBL cells provided a more robust response with an average IC_{50} of 89.15 ± 3.38 µg/mL.
- Only trials which had an $R^2 > 0.85$ for the Hill fit and low % difference between the two columns containing the vehicle controls (VC1 and VC2 in Figure 1) were included to limit plate variability.

- The average absorbance displayed in the vehicle control wells varied by trial, but overall was approximately the same between the two cell lines, suggesting similar growth, final cell density and similar uptake of the NR dye across the two cell lines.