Neutral Red Uptake Cytotoxicity Assay in A549 Cells Under Different Culture Conditions: Part of Assay Evaluation for Potential Use in Direct Aerosol In Vitro Testing

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71st Tobacco Science Research Conference, November 28 - December 1, 2017, Bonita Springs, FL, USA

This poster may be accessed at www.altria.com/ALCS-Science

Abstract

OECD guideline (TG129) recommends mouse fibroblasts (3T3) and normal human epidermal keratinocytes (NHK) for performing in vitro cytotoxicity assays, but also mentions limitations of these cells, e.g., having little to no metabolic capacity or possibility of inappropriate interpretation of data if these cells are unrelated to the target organ of interest. Considering the primary target organ for inhalable products, we conducted this study to evaluate the suitability of a human derived alveolar cell line A549 for the neutral red uptake (NRU) assay under various testing conditions. First, positive control sodium lauryl sulfate (SLS) and dimethyl sulfoxide (DMSO)-extracted cigarette smoke total particulate matter (TPM) were tested for cytotoxicity in A549 and 3T3 under submerged condition. Secondly, A549 were grown at the air-liquid interface (ALI) and assessed for cytotoxicity following 24 and 48 h of treatment with SLS in media supplied from basal side containing 0% or 5% serum. Dose-dependent cytotoxicity was observed in 3T3 and A549 in response to both SLS and TPM and the corresponding IC_{50} were estimated. Under submerged conditions containing serum, A549 displayed lower IC_{50} (SLS) and higher IC_{50} (TPM) in comparison to the values of mouse 3T3 cells, suggesting species-dependent differences in sensitivities. No significant difference in cytotoxicity was observed in A549 cells between submerged and ALI cultures in response to SLS. Enhanced cytotoxicity was evident in A549 cells exposed to SLS under serum free environmental conditions. In summary, (1) A549 cells could be effectively used in the TG129 NRU assay in submerged as well as ALI conditions and (2) media composition, such as presence of serum, should be carefully controlled especially when using human-derived cells such as A549.

Introduction

In vitro cytotoxicity assays are widely used as part of regulatory toxicity testing as an early screening for possible health hazards. The NRU basal cytotoxicity assay procedure is based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye,[1] which is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. Cytotoxicity is commonly expressed as IC_{50} (the concentration inducing 50% reduction in viability).

Objectives

1. Evaluate the suitability of A549 alveolar cells in comparison to BALB/C 3T3 cells for their use in the regulatory Neutral Red Uptake (NRU) cytotoxicity assay.
2. Compare the cytotoxicity in A549 cells at Air Liquid Interface (ALI) and standard submerged conditions.

Conclusions

- Dose-dependent cytotoxicity was observed in 3T3 and A549 in response to both SLS and TPM.
- Under submerged conditions with serum, human A549 displayed more sensitivity to SLS-mediated cytotoxicity, but less sensitivity to TPM-mediated cytotoxicity in comparison to mouse 3T3 cells, suggesting cell and species-dependent differences in sensitivities.
- Similar cytotoxic potencies were observed in A549 cells between submerged and ALI conditions in response to SLS.
- Enhanced cytotoxicity was evident in A549 cells exposed to SLS under serum free environmental conditions.
- A549 cells could be effectively used in the OECD 129 NRU assay in submerged as well as ALI conditions.
- Media composition, such as the presence of serum, should be carefully considered especially when using human-derived cells such as A549.

Materials & Methods

Materials

Test Article: TPM collected from mainstream cigarette smoke (3R4F) at stock concentration of 35 mg/mL in DMSO generated under Canadian Intense conditions according to Health Canada T-1152.[2]

Control Articles: Vehicle control: DMSO. Final concentration was 0.5% (v/v). Positive control: SLS. Stock solution was prepared in water at 5 mg/mL (6.8–100 µg/mL).

Cells: BALBc/3T3, clone 31: Murine embryonic fibroblasts derived cell line was procured from ATCC (clone A31, Lot no.61633509).
A549: Human adenocarcinoma derived alveolar type II cell line was obtained from ATCC (cell Lot no. 091316c4).

Media: BALBc/3T3: DMEM (Dulbecco's Modified Eagles medium) supplemented with 10% newborn calf serum (NCS), 100 IU/mL penicillin and 100 µg/mL streptomycin.
A549: Complete F-12 K medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin.

Cytotoxicity Assay

BALB/c 3T3 and A549 cells-submerged (OECD 129): Day prior to treatment with the test and control articles, exponentially growing cells were seeded into wells of a 96-well plate at a density of approximately 3000 cells/well in serum supplemented respective media and were incubated overnight in a humidified incubator at 37 ± 1°C in an atmosphere of 5% ± 1% CO_2 in air. The confluency of the cells at the time of treatment was ~50%. Following 24 h of culture, cells were treated with controls or test articles in medium with or without serum for 48 h. The doses of TPM were 20, 40, 60, 80, 100, 120, 140, and 160 µg/mL. The concentration of DMSO was held constant at 0.5 % (v/v).

A549 Cells at Air Liquid Interface: Cells were seeded at a density of 50,000 cells on pre-conditioned inserts with microporous membranes (pore size = 0.4 µm, ThinCert™, Greiner Bio One International AG, Kremsmünster, Austria) as submerged phase and cultured for 24 h in a humidified incubator at 37 ± 1°C in an atmosphere of 5% ± 1% CO_2 in air prior to use in the NRU assay. The media was then removed from both the apical and basal side and was replaced with fresh medium with or without serum containing control or test articles only on the basal side for either 24 h or 48 h. The apical side of the membrane, where the cells are seeded, is at the air lifted phase and the basal side of the membrane is in contact with the media. This configuration is termed ALI.

Neutral Red Uptake Assay

Following treatment, media was removed and replaced with medium containing NR dye. Cells were incubated with the NR dye for ~3 h at 37 ± 1°C in a 5% ± 1% CO_2 atmosphere, following which the monolayer was washed once with PBS. Desorb solution was added to extract the NR dye, followed by measurement of absorption at 540 nm.

Data Analysis

The average, standard deviation, and relative standard deviation (%RSD) were calculated for the uncorrected, blank corrected, and relative absorbance for each group of 6 replicate wells on each plate. The average normalized absorbance values at each test article dose were used to construct a dose-response curve for each assay. IC_{50} values were calculated by nonlinear regression fitting to the Sigmoidal dose-response curve using GraphPad Prism statistical software (version 5.0).

References

1. Borenfreund, E. and Puerner, J.A. (1985) Toxicity determination in vitro by morphological alterations and neutral red absorption. Toxicology Letters 24:119-124.
2. Health Canada Official Method T-502 (Second Edition, 2004-11-01), Neutral Red Uptake Assay for Mainstream Tobacco Smoke.
3. OECD. (2010) OECD Guidance Document on Using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests.

Results

Table 1. NRU assay response between A549 and 3T3 cells (with 0% and 5% serum during 48 h exposure)

Treatment	IC_{50} (µg/mL)			
	No Serum		5% Serum	
	3T3	A549	3T3	A549
SLS	13.30 ± 3.47 (n=6)	9.56 ± 0.38 (n=9)	75.1± 0.2 (n=7)	49.4 ± 0.43 (n=7)
TPM	21.89 ± 6.72 (n =12)	28.35 ± 3.85 (n= 20)	28.0 ± 3.5 (n =5)	108.0 ± 15.5 (n= 5)

Table 2. NRU assay with A549 cells cultured under submerged conditions vs. at ALI (0% and 5% serum)

Treatment	IC_{50} (µg/mL)			
	No Serum		5% Serum	
	A549-Submerged	A549-ALI	A549-Submerged	A549-ALI
SLS	9.56 ± 0.38(n=9)	12.17 ± 2.7 (n=5)	49.4 ± 0.43 (n=7)	58.4 ± 3.0 (n=2)

Table 3. NRU assay at ALI in A549 cells at 24 h vs. 48 h (with 0 and 5% FBS)

Treatment	IC_{50} (µg/mL)			
	No Serum		5% Serum	
	A549-24 h	A549-48 h	A549-24 h	A549-48 h
SLS	14.5 ± 0.9 (n=5)	12.17 ± 2.7 (n=5)	66.0 ± 0.5 (n=2)	58.4 ± 3.0 (n=2)

Figure 1. Comparison of cytotoxicity of SLS and TPM in 3T3 and A549 cells grown under submerged conditions (without serum)

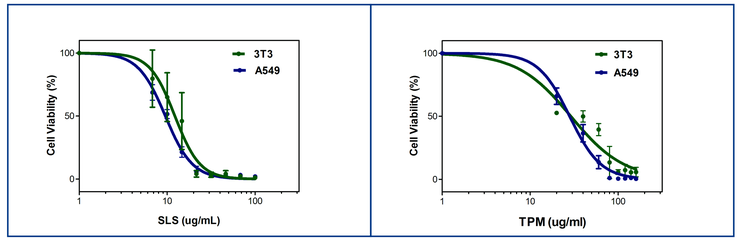

Figure 2. Comparison of cytotoxicity of SLS and TPM in 3T3 and A549 cells grown under submerged conditions (with serum)

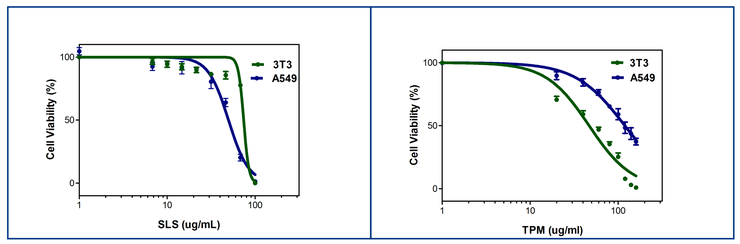

Figure 3. Cytotoxicity of SLS in A549 cells grown under submerged conditions and at ALI (left panel without serum and right panel with serum)