Genotoxicity Evaluation of Tobacco and Nicotine Delivery Products
Part 2: *In Vitro* Micronucleus Assay

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Abstract

In vitro studies have been widely used to support toxicological evaluations of chemicals and complex mixtures including cigarette smoke. In this study a variety of test matrices from tobacco and nicotine delivery products were assessed against a 3R4F reference cigarette using the *in vitro* micronucleus assay (IVMN). Assays were conducted in general accordance to OECD Guideline 487 and ICH S2 (R1) guidance. Samples were also assessed using the mouse lymphoma assay (Part 1).

3R4F total particulate matter (TPM) was first assessed with CHO, V79 (both rodent) and TK6 lymphoblastoid (human) cell lines with 3hr exposures ±S9 metabolic activation and extended -S9 treatments with/without a 1.5-2 cell cycle length recovery period at doses up to 500µg/mL. CHO, V79 and TK6 cells gave varied positive responses, with V79 being most responsive. The extended recovery treatment increased assay sensitivity for CHO and V79 cells; this was less clear in human TK6 cells. V79 cells were taken forward for further assessments.

3R4F TPM was compared against pad-collected aerosol matter generated from a commercial electronic nicotine delivery system (ENDS), a commercial e-liquid, and TPM from a commercial tobacco-heating product (THP) using the same treatment schedules described above.

Across all treatment regimens with V79 cells, clear negative responses were observed for the e-liquid, ENDS and THP samples, while 3R4F elicited a clear positive response. The most potent 3R4F responses were observed following extended treatment -S9 with recovery, suggesting this to be a more appropriate treatment schedule for the assessment of tobacco and nicotine product test matrices. Based on the results of this study the IVMN assay can be used effectively for the assessment of these test matrices.

Introduction

Next generation tobacco and nicotine products (NGPs) which are comprised of THP and ENDS have evolved significantly over the last few years and are gaining consumer acceptability. Available *in vitro, in vivo* and clinical data indicate these products may possess significantly lower biological activity when compared to cigarette smoke [1], suggesting these products may be of reduced harm, when compared to cigarette smoking, and offer a potential opportunity for global harm reduction [2].

In this study, conducted in two phases, the *in vitro* micronucleus (IVMN) cytokinesis block assay was used for the assessment of NGPs with the aim to identify optimal assay parameters.

Phase 1: To establish optimum assay conditions by comparing three different cells lines (CHO, V79 and TK6) on their ability to respond to cigarette smoke TPM with the inclusion of two extended treatment regimens (treatment for 1.5 to 2 cell cycles with or without a 1.5 to 2 cell cycle recovery period).

Phase 2: To use the optimized conditions and cell type determined in Phase 1 to compare the activity of NGP products against cigarette smoke TPM.

This study is Part 2 of a two-part series where the same test articles and TPM preparations were compared in both the mouse lymphoma assay (MLA) and the IVMN assay, in a coordinated testing approach.

Materials and Methods

Sample Preparation:

TPM and e-liquid

Samples	Preparation Process	Extraction Process
3R4F	HCI smoking regimen (55 mL puff volume, 2 sec puff duration, 30 sec puff interval, 100% vent blocking)	In DMSO: 20 mg/mL
THP	Modified HCI puffing regimen (no vent blocking)	In DMSO: 50 mg/mL
ENDS	CRM81 puffing regimen (55mL puff volume, 3 sec puff duration, 30 sec puff interval)	In DMSO: 50 mg/mL
e-liquid	Diluted in DMSO to desired dosing concentrations	DMSO

Cell Culture & Treatments:

- Cells initiated from frozen stock 5-7 days prior to treatment, sub-cultured accordingly to maintain exponentially growing cells. Cells sub-cultured at suitable cell densities 1 day prior to treatment.

Cell Line	CHO	V79	TK6
Origin	Chinese Hamster		Human
p53 status	Mutant		Wild-type
Supplier	ECACC		
Cell cycle time	12-13 hrs	9-12 hrs	15-17 hrs
Culture	McCoy's	DMEM	RPMI 1640 +
Serum	10% heat inactivated fetal calf serum		

Cell Line	Treatment + Recovery (hrs)	S9 Addition	Test Article Addition (hrs)	Removal of Test Article (hrs)	Cyto B Addition (hrs)	Harvest Time (hrs)
CHO and V79*	3+21*	+/-	0	3	3	24
	24+0	-	0	-	0	24
	24+24*	-	0	24	24	48
TK6	3+27	+/-	0	3	3	30
	30+0	-	0	-	0	30
	27+27	-	0	27	27	54
*Cell Line and Treatments Utlized in Phase 2						

- Slides were prepared and stained with 12.5 mg/mL Acridine orange
- Cytotoxicity (%) expressed via relative replication index (RI) (OECD 487 [3])
- 2000 (Phase 1) or 8000 (Phase 2) BN cells/dose were scored (manually) for micronuclei
- Statistics: one sided Fisher's exact test (Phase 1), Wilcoxon Rank Sum test (Phase 2)

References:

1. Ay, O., Kacker, A. Do electronic cigarettes impart a lower potential disease burden than conventional tobacco cigarettes? Review on e-cigarette vapor versus tobacco smoke. Laryngoscope 2014;124 (12): 2702-2706.
2. McNeill A, et al. E-cigarettes: an evidence update. A Report by Public Health England 2015. https://www.gov.uk/government/publications/e-cigarettes-an-evidence-update (Accessed 27th April 2018)
3. OECD (2016). Genetic Toxicology: OECD Guideline for the testing of chemicals. Guideline 487: *In vitro* mammalian cell micronucleus test.

Conclusions

- In this study, 3R4F TPM elicited an induction of MNBN cells in all three cell lines tested.
- V79 cells were observed to be the most sensitive cell line to cigarette smoke TPM, with a clear and reproducible response, as compared to CHO or TK6 cells.
- The most potent response observed in V79 cells with 3R4F TPM was following the extended treatment with recovery (treatment for 1.5 to 2.0 cell cycles with 1.5 to 2.0 cell cycle recovery), indicating that this treatment regimen would be useful in future product testing.
- Under the optimized assay parameters (V79 cells, extended treatment with recovery), THP and ENDS test matrices (TPM & e-liquid) were all negative in the induction of micronuclei, at concentrations far exceeding those of cigarette smoke and up to the maximum deliverable dose.

This is Part 2 of a two-part series where the same test article preparations were tested in both the MLA (not shown here) and the IVMN assay. Both assays demonstrated similar results, further validating the use of either the MLA or IVMN in the assessment of these product types.

Results

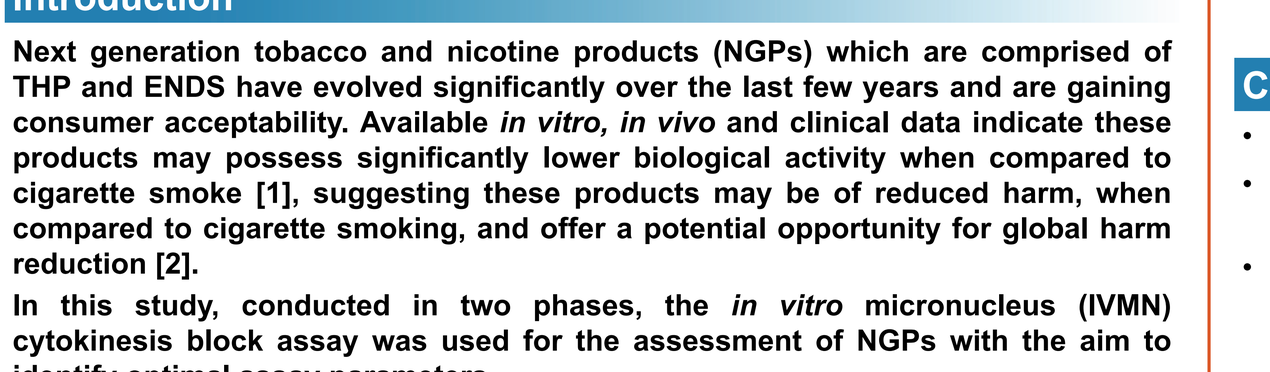

FIGURE 1: Exposure of three cell types, CHO (A), V79 (B) and TK6 (C), with pad-collected TPM (µg/mL) from a 3R4F combustible reference cigarette under the treatment schedules indicated. Bar charts show MNBN (micro-nucleated binuclear cell) frequency of the individual replicate cultures (mean MNBN frequency indicated by ♦). The level of toxicity, calculated as a % reduction in replication index (RI) relative to concurrent vehicle controls, is indicated with linear plots (right Y-axis). The dashed line (---) represents the upper 95th percentile of the historical control range (HCR) for each treatment and cell type.

*p < 0.05; statistical significance compared to vehicle control; one-tailed Fisher's exact test.

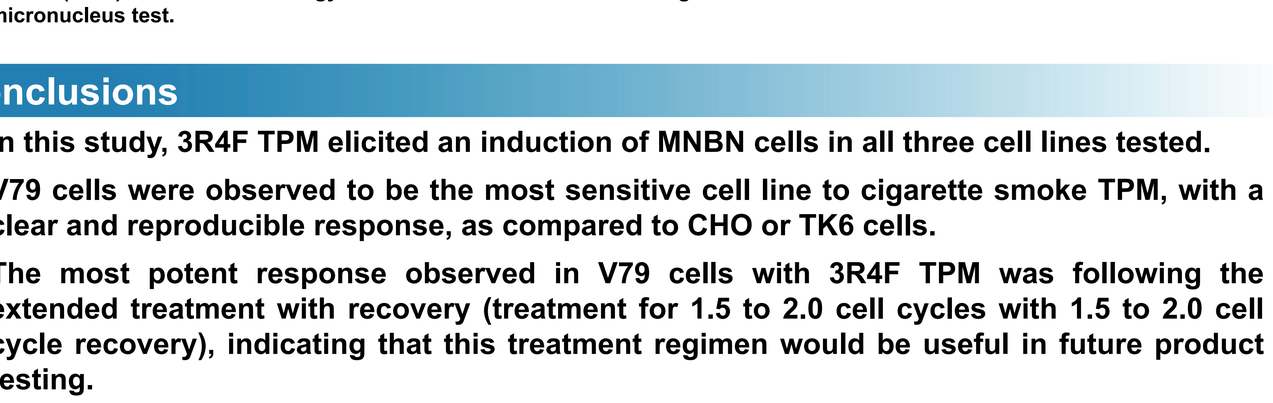

FIGURE 2: Exposure of V79 cells to a commercial e-liquid or pad-collected TPM (µg/mL) from a commercially available ENDS, THP or 3R4F combustible reference cigarette under three treatment schedules (A-C). Bar charts show MNBN frequency of the individual replicate cultures (mean MNBN frequency indicated by ♦). The level of toxicity, calculated as a % reduction in replication index (RI) relative to concurrent vehicle controls, is indicated with linear plots (right Y-axis). The dashed line (---) represents the upper 95th percentile of the historical control range (HCR) for each treatment.

*p < 0.05; statistical significance compared to vehicle control; Wilcoxon Rank Sums test.

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