Assessment of *in vitro* toxicities demonstrated by total particulate matter (TPM) generated from current market and research reference standard cigarettes using the Bhas 42 Promotor Cell Transformation Assay

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Abstract

The Bhas 42 cell transformation assay (CTA) has been used to complement standard *in vitro* testing of tobacco products for understanding downstream biological implications of genetic toxicological changes related to tumor promotion. Investigators have typically published CTA results showing tumor promotion by the combustible Kentucky Reference (KY) cigarette 3R4F compared to the lack of tumor promotion from next generation/non-combusted tobacco products. Although it is more relevant to benchmark the tumor promotion activity of new or potentially reduced risk tobacco products with currently marketed cigarette products rather than reference standards, few results/comparisons have been published. In the present study, we used the CTA assay (OECD 231) to determine the tumor promotion potential of four combustible cigarettes, including two leading US market products, Marlboro Gold Pack Box and Newport Box, and two KY reference cigarettes, 3R4F and 1R6F. Total Particulate Matter (TPM) was collected from the test cigarettes according to Health Canada Intense (HCI) smoking regimen parameters. Based on the preliminary growth assessment assay data, the TPM concentrations selected for testing ranged from 5-125 µg TPM/mL for all four test items. Under the conditions of this assay, all test items were positive in the promotion assay for cell transforming activity, with the lowest effective concentration of each test item ranging from 5 to 25 µg TPM/mL. These data demonstrate that there was little variability among the test item responses, and each product induced a dose-related response in the promotion assay. In summary, two current leading market combustible products yielded tumor promotion activity that was consistent with responses observed for KY cigarettes suggesting that they could be used as combustible cigarette comparators in future Bhas 42 assays.

Introduction

One of the most prevalent human health risks associated with smoking of combustible cigarettes is lung cancer. Although guidance (CORESTA, 2019; OECD: TG 487; 471) for *in vitro* testing of tobacco products has traditionally advised use of the Ames *Salmonella typhimurium* mutation and micronucleus assays (e.g. Aufderheide and Gressmann, 2008; DeMarini et al., 2008; Thorne et al., 2015), to detect cancer-related biological mechanisms, these tests only address disease initiating events such as gene mutation or other DNA damage. In order to more fully understand the potential carcinogenic risk of new tobacco products, a number of investigators have established that the Bhas 42 CTA assay is useful in supplementing traditional nonclinical testing approaches by its ability to detect both genotoxic and non-genotoxic carcinogens that may be involved in tumor promotion (Weisensee et al., 2013, Thorne et al., 2015, Breheny et al., 2017, Sasaki et al., 2011). Thus, the Bhas 42 cell transformation assay promotor protocol can be used to support a "weight of evidence" based *in vitro* testing strategy for tobacco products.

Product innovation in the tobacco industry has led to the development of new/alternative products that may not present the same human health risks of more traditionally marketed products. In its draft guidance for Modified Risk Tobacco Product Applications (MRTPA), the FDA Center for Tobacco Products (CTP) describes a modified risk product as a tobacco product that is "sold or distributed for use to reduce harm or the risk of tobacco-related disease associated with commercially marketed tobacco products". Thus, an important aspect of modified risk tobacco product development and subsequent application for regulatory authorization in the US is to compare relevant disease-related scientific endpoints of the new product with those of a currently marketed tobacco product. To date, however, the majority of published Bhas 42 CTA studies on new or (potentially) modified risk tobacco products have used a generic research reference standard cigarette as a comparator rather than a marketed product, as recommended by the CTP for MRTPAs.

In the present study, we sought to address the question of whether commercially-marketed tobacco products would yield positive results in the Bhas 42 assay in a similar manner to that of research reference combustible cigarettes, thereby establishing an initial data set by which the use of marketed combustible products may be utilized as positive controls in the Bhas 42 promotor assay for future MRTPAs. In addition, we concurrently tested both the 3R4F and 1R6F reference combustible cigarettes to determine if there was any notable variation in the response. The study was conducted according to Bhas 42 CTA OECD draft guidance (2017), with triplicate replicates, and an initial cell growth assay was performed for each replicate.

References

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4. Breheny, D et al., 2017. Comparative tumor promotion assessment of e-cigarette and cigarettes using the in vitro Bhas 42 cell transformation assay. *Environmental and Molecular Mutagenesis,* 58: 190-198.
5. Organisation for Economic and Cooperative Development. 2017. *Guidance Document on the in vitro Bhas 42 cell transformation assay. Series on Testing and Assessment.* No. 231

Material and Methods Figures

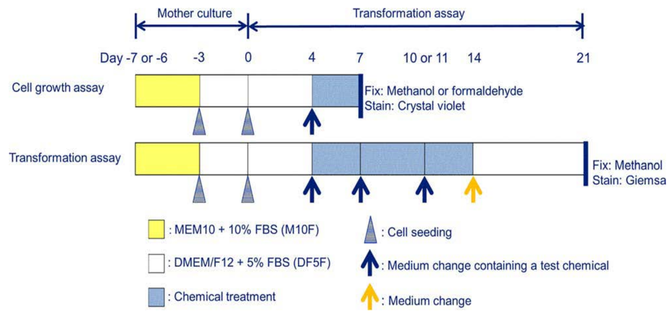

Figure 1: Promoter Transformation Assay and Parallel Cell Growth Assay; image adopted from OECD Series on Testing and Assessment. No. 231 Guidance Document on the in vitro Bhas 42 cell transformation assay (2017).

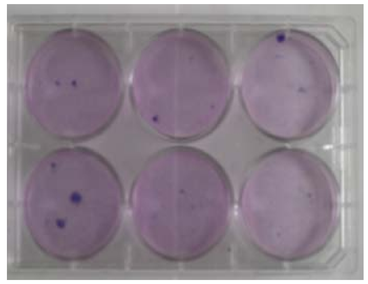

Figure 2: Assay is performed in 6 well plates (2 mL: 14,000 cells/well), 3 wells per concentration for the preliminary toxicity assay and the concurrent cell growth assay and 6 wells per concentration for the transformation assay

Results

Table 1: Number of transformed foci quantified in each of three replicate promotor cell transformation assay experiments following exposure to TPM from combustible cigarettes.

TPM Dose Level	Marlboro Gold Pack Box			Newport Box			1R6F Reference			3R4F Reference		
	Foci Counts			Foci Counts			Foci Counts			Foci Counts		
	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0 µg/mL (Vehicle)	2.3	1.7	7.3	2.5	1.0	1.8	4.2	5.5	5.7	5.3	3.3	3.3
5 µg/mL	*5.5	*4.2	*14.8	*5.2	3.0	3.3	*10.8	*11.2	7.2	6.3	*8.5	*6.7
10 µg/mL	*7.0	*5.2	*24.0	*6.0	*4.5	*5.5	*18.2	*14.3	*9.8	*10.0	*10.0	*11.3
25 µg/mL	*8.8	*9.2	*33.5	*10.5	*9.8	*13.8	*23.7	22.3	20.8	15.8	16.7	17.3
50 µg/mL	*12.8	*14.8	*27.7	*12.5	*15.5	*17.0	*24.7	*26	*26.2	*24.5	*23.2	*25.0
75 µg/mL	*14.2	1.8	*14.0	*5.0	*9.5	*10.7	*18.7	*18.3	*17.7	*14.8	*14	*16.2
100 µg/mL	*8.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
125 µg/mL	*9.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
0.2% DMSO	1.3	0.7	5.7	2.7	2.3	3.3	3.3	2.3	3.7	4.5	5.8	5.2
50 ng/mL TPA	*5.2	*6.8	*15.7	*6.5	*5.2	*6.3	*14.5	*15.2	*13.2	*11.8	*8.8	*10.5

TPA=12-O-Tetradecanoylphorbol-13-acetate
* Significantly greater (p<0.05) than # of foci counted following treatment with 0.5% DMSO vehicle control.

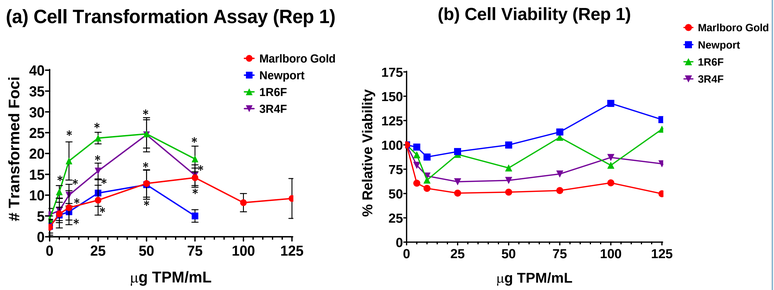

Figure 3: Graphs represent the (a) mean foci response and (b) cytotoxicity for the first assay replicate, * indicates significantly greater (p<0.05) than # of foci counted following treatment with 0.5% DMSO vehicle control.

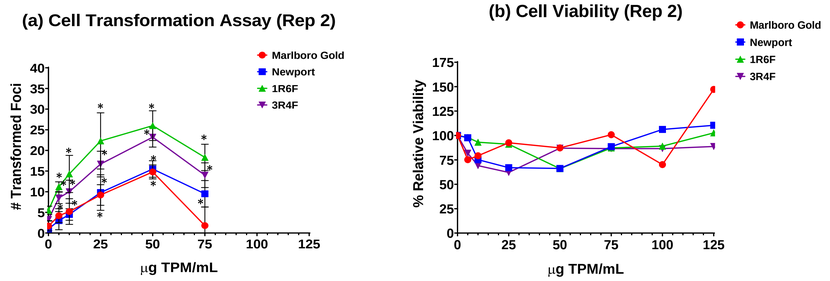

Figure 4: Graphs represent the (a) mean foci response and (b) cytotoxicity for the second assay replicate, *indicates significantly greater (p<0.05) than # of foci counted following treatment with 0.5% DMSO vehicle control.

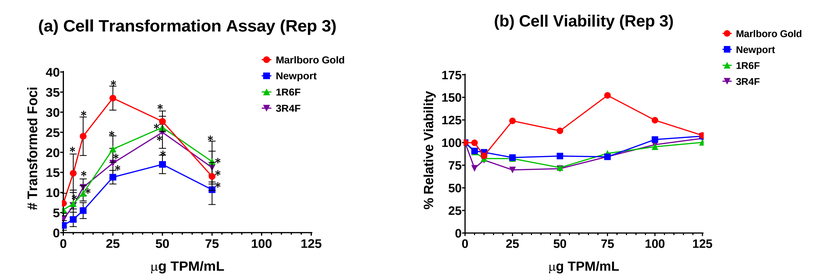

Figure 5: Graphs represent the (a) mean foci response and (b) cytotoxicity response for the third assay replicate, * indicates significantly greater (p<0.05) than # of foci counted following treatment with 0.5% DMSO vehicle control.

Materials and Methods

Generation of the Total Particulate Matter (TPM):

- On three separate occasions, each of the four combustible cigarette test articles were smoked using a rotary smoking machine according to Health Canada Intense (HCI) regime parameters of 55 mL puff volume, 30 second interval, 2 second duration (55/30/2), with a bell-shaped puff profile and fully blocked ventilation holes. On each occasion, approximately 600 mg TPM from each test article was collected on a pre-conditioned, pre-weighed 92mm Cambridge filter pad (CFP) and the TPM was extracted in dimethylsulphoxide (DMSO) at a concentration of 40 mg TPM/mL.
- TPM extracts from each test article was aliquoted (1 mL) into sterile cryogenic vials and stored in a cryofreezer (upper temperature of -70°C) until required for testing.
- The three separate batches of TPM extract from each test article were used to assess the four products on three separate (independent replicate experiment) occasions in the Bhas 42 promoter assay.

Preparation of Cell Stock

- Bhas 42 cells were cultured in an incubator under standard conditions (5 ± 1% CO_2 at 37.0 ± 1.0°C with ≥ 85% humidity).
- The cells were expanded and cryopreserved in M10F (minimal essential medium with 10% fetal bovine serum and 1% penicillin/streptomycin) prior to use in the CTA.

CTA Promoter Transformation Assay and Parallel Cell Growth Assay:

Cell growth assay (Figure 1)

Day -6 or -7: Frozen stock cells (0.5 x 10^6 cells) were thawed and cultured in 20 to 50 mL of M10F in T-75 flasks, at a volume of 10 mL per flask.

Day -3: Cells at 40 to 70% confluence were trypsinized and re-suspended in Dulbecco's modified Eagle's medium: F12 (DMEM:F12) with 5% fetal bovine serum and 1% penicillin/streptomycin (DF5F) at 0.7 to 1.0 x 10^4 cells/mL. The cell suspension was transferred at a volume of 10 mL per T-75 flask.

Day 0: Cells at 40 to 70% confluence were trypsinized and re-suspended in DF5F at 7000 cells/mL. The cell suspension was distributed into each well of 6-well plates at a volume of 2.0 mL (~14000 cells/well). After seeding the cells, the plates were incubated at standard conditions (5 ± 1% CO_2 at 37.0 ± 1.0°C with ≥ 85% humidity).

Day 4: Cells (three wells per treatment group) were treated with test article TPM extract (0.25-250 µg/mL), vehicle or positive control.

Day 7: Cells were fixed and stained followed by solvent extraction of the retained stain, as previously described (Breheny et al., 2017). Growth rates relative to the solvent/vehicle control culture were calculated. The doses for the promoter transformation (definitive) assay were selected with the highest dose having approximately 50% relative toxicity.

Transformation Assay - Promoter Protocol

The initial steps for the transformation assay promotor protocol are similar to those of the cell growth assay, with the following exceptions:

Day 0: nine wells were seeded per treatment group

Day 4: Test sample (5-125 ug/mL TPM extract), blank control, positive control (12-O-tetradecanoylphorbol-13-acetate (TPA)), and vehicle control treatments were performed (nine wells per treatment group).

Day 7: Media was replaced with fresh media containing respective test sample concentrations, positive controls or vehicle controls.

Day 10 or 11: Media was replaced with fresh media containing respective test sample concentrations, positive control or vehicle controls.

Day 14: Media was replaced with 2.0 mL of fresh DF5F.

Day 21: Cells were fixed in methanol for ~ 10 minutes and stained with freshly prepared 5% Giemsa solution for ~ 15 minutes, rinsed in tap water and air-dried. Plates were scored and results evaluated as previously described (Breheny et al., 2017).

Summary and Conclusions

- This study shows that all four combustible test articles exhibited a positive response in the Bhas 42 tumor promotion assay by generating a statistically significant increase in the number of transformed foci when compared to the vehicle control. These results were reproducible across 3 replicate independent experiments.
- The results of this study demonstrate that the commercially marketed tobacco products yielded positive results in the Bhas 42 promotor assay in a similar manner to that of research reference combustible cigarettes.
- The results of this study also suggest that the commercially marketed tobacco products from this study could be included as positive controls in future Bhas 42 promotor assay studies.
- No dose from any TPM tested resulted in a reduction of cell viability of > 50%.