Assessment of the Cell Transformation Assay Using Traditional and Next Generation Tobacco Products

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Abstract

Given its ability to simulate the oncogenic stages of *in vivo* initiation and promotion of cell transformation leading to carcinogenesis, the *in vitro* Bhas 42 cell transformation assay (CTA) represents a useful method for assessing the (non-genotoxic mediated) carcinogenic potential of chemicals and complex mixtures that would not be detected using traditional *in vitro* genetic toxicology assays such as Ames or *in vitro* micronucleus. Upon exposure to transformation-inducing stimuli, the contact-inhibited Bhas 42 cells may change morphologically to form discrete, altered colonies (transformed foci) on top of the confluent cell monolayer. Comparable to the features of tumor transformation *in vivo*, transformed foci are characterized by deep basophilic staining, random cell orientation, increased cell density, and invasive growth into the surrounding cell monolayer (OECD, 2016).

The potential transformation promotion activity of TPM (total particulate matter) test samples prepared from combustible cigarettes, a heated tobacco product (HTP), and an electronic nicotine delivery system (ENDS) product were individually assessed using the CTA assay. The purpose of these studies was two-fold: (1) to assess new contract research laboratory vendors for performing the CTA promotion test and (2) to demonstrate its reproducibility, reliability, and fit-for-purpose by testing the transformation promoting potential of a variety of traditional and next generation tobacco products.

Results reproducibly showed a transformation promoting effect of the combustible test sample. Conversely, neither the ENDS nor the HTP test samples showed transformation promotion activity. Furthermore, the consistency of the assay results demonstrated by the vendors verified their ability to reproducibly perform the CTA. Taken together, these results add to the weight of evidence from multiple studies showing that HTP and ENDS products are less toxic compared to traditional combustible products. These results demonstrated that CTA is reproducible and reliable and could be a useful tool for the assessment of next generation tobacco products.

Introduction

One of the most prevalent human health risks associated with smoking combustible cigarettes is lung cancer. Although regulatory guidance (OECD: TG 471; 487) for *in vitro* testing of tobacco products has traditionally advised the use of the Ames *Salmonella typhimurium* mutation and micronucleus assays to detect cancer-related biological mechanisms, these tests only address cancer initiating events such as gene mutation or other DNA damage. The Bhas 42 cell transformation assay has emerged as a useful tool for identifying carcinogenic induction by chemicals and complex mixtures resulting from non-genotoxic mechanisms. Moreover, the Bhas 42 CTA has become widely used in studies investigating the potential carcinogenic risk posed by new tobacco products (Weisensee et al., 2013, Breheny et al., 2017, Sasaki et al., 2011).

Product innovation in the tobacco industry has led to the development of new/alternative tobacco or nicotine products that may not present the same potential human health risks of more traditionally marketed products. Studies like the CTA provide important information on potential disease relevant scientific endpoints for new versus current marketed products. The present study sought to identify and validate at least two vendors to conduct the Bhas 42 cell transformation assay. The primary goals of these validation efforts were to verify results would be qualitatively consistent between the two vendors as well as comparable over a range of next generation tobacco products. The study was conducted according to Bhas 42 CTA OECD draft guidance (2016), with an initial cell growth assay followed by a promoter assay and cell growth assay.

Material and Methods

Generation of the Total Particulate Matter (TPM): All puffing/smoking was done on a rotary smoking machine. The following smoking/puffing parameters were used during this study:

Test Item	Smoking Regime	Puff Volume (mL)	Puff Duration (s)	Puff Interval (s)	Puff Profile	Vent Blocking	Puffs per Collection*	Device Orientation
Marketed Combustible Comparator	HCI	55	2	30	Bell	100%	to butt mark	N/A
Marketed HTP	HCI	55	2	30	Bell	0%	13	N/A
Marketed ENDS	CRM81	55	3	30	Square	N/A	100	horizontal

Notes: * Five (5) clearing puffs were taken at the end of the rotary puff run

** Pre-heat time for HTP device – 30s (Pre-heat time ≡ time lag between device activation and collection of the first puff)

Conditioned HTP, combustible tobacco products (ISO 3402, 1999) and ENDS emissions were generated using an automated constant volume smoking machine. Compounds of interest were trapped using a 92mm conditioned glass fiber filter disc (pad). TPM was collected on the filter pad to allow preparation of extracts in DMSO at the concentrations of 40 mg/mL for the combustible and 100 mg/mL for the HTP & ENDS products.

Promoter Transformation Assay and Parallel Cell Growth Assay to confirm dose range for the promotion assay in a non-cytotoxic range:

Cell growth assay (Figure 1)

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

Day -3: Cells at 40 - 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 - 70% confluence were trypsinized and re-suspended in DF5F at 7,000 cells/mL. The cell suspension was distributed into each well of 6-well plates at a volume of 2.0 mL (~14,000 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 - 500 µg/mL), vehicle or positive controls.

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 (Figure 1)

Day 0: Nine wells were seeded per treatment group (three wells for parallel cell growth assay).

Day 4: Test sample (0.25 - 500 µg/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 or vehicle controls.

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

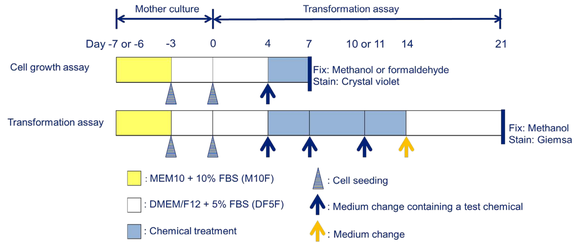

Figure 1: Promoter Transformation Assay and Parallel Cell Growth Assay (Breheny, D. et al., 2017)

References

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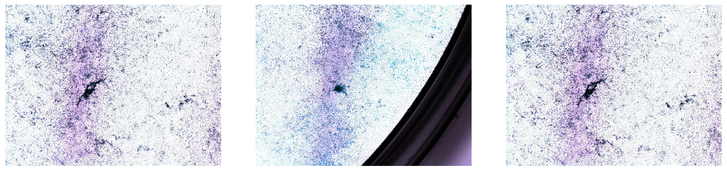
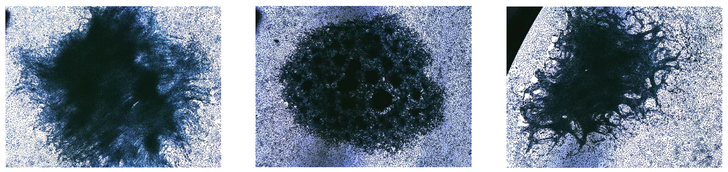
4. Organisation for Economic and Cooperative Development. 2016. Guidance Document on the *in vitro* Bhas 42 cell transformation assay. Series on Testing and Assessment. No. 231
5. ISO 3402 (1999). Tobacco and tobacco products – Atmosphere for conditioning and testing (4th edition)
6. OECD (2016), Test No. 487: In Vitro Mammalian Cell Micronucleus Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264264861-en
7. OECD (2020), Test No. 471: Bacterial Reverse Mutation Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264071247-en

Results

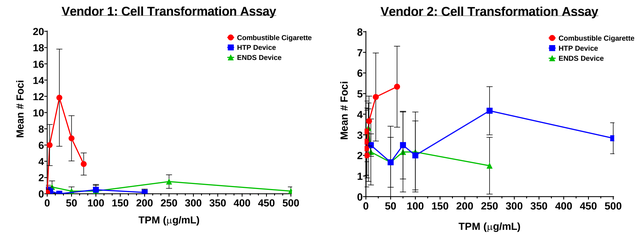
Negative Foci: Example Images

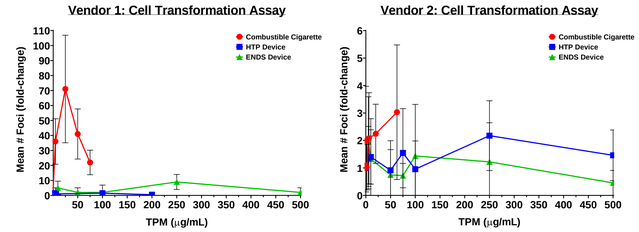


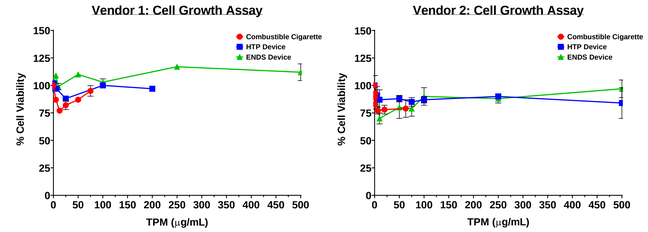
Positive Transformed Foci: Example Images



Bhas 42 Cell Transformation Assay Results







Summary and Conclusions

- **This study shows that results for the combustible product from both vendors 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.**
- **For both vendors, assay results showed no statistically significant increase in the number of transformed foci for the HTP and ENDS products.**
- **The results were qualitatively comparable between the two vendors for each product tested.**

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