Cellular Transformation activity of different tobacco products in the Bhas 42 cell assay

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2016 CORESTA Congress
9 – 13th October 2016

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1. Introduction and Objectives

1.1 Introduction

Carcinogenesis has been described as a multi-stage process comprising of initiation, promotion and progression. Smoking is a cause of serious disease in smokers, including lung cancer. Chemical carcinogens can be categorised by their mode of action into either genotoxic carcinogens or non-genotoxic carcinogens.

The Cellular Transformation assay is an *in vitro* assay which replicates the initiator and promotor stages of carcinogenicity[1]. Additionally, this assay can detect both genotoxic and non-genotoxic carcinogens. In 2016, the OECD issued a guidance document for the Bhas-42 Cellular Transformation assay and suggested that this assay will be used as part of a testing strategy and/or in a weight of evidence approach in predicting carcinogenic potential[2].

Determining the carcinogenic potential of a tobacco product ingredient is a key component of a stewardship assessment. Recently, the Bhas 42 Cellular Transformation assay has been used to assess the carcinogenic potential of particulate matter generated from American blended cigarettes[3]. The assay was concluded to have a good sensitivity and precision when assessing Total Particulate Matter (TPM).

1.2 Objectives

The aim of the study was to assess the initiation and promotion potential of TPM from different tobacco products (factory manufactured cigarettes with and without additives) in the Bhas-42 Cellular Transformation assay. The ability of the assay to distinguish a concentration response for different types of TPM was also investigated.

2. Materials and Methods

2.1 Test Substance Preparation

Total Particulate Matter was generated from three different tobacco products:

- American Blended cigarette (AB)
- Additive free American Blended cigarette (a.f.AB)
- Virginia blended cigarette (VB) – also additive free

The mainstream smoke from cigarettes was generated in accordance with ISO 3308. The Total Particulate Matter was collected on Cambridge filter pads (up to 600mg per pad) and extracted with DMSO, achieving a TPM concentration of up to 50mg/ml. A solvent control (DMSO) and blank were included in each experiment.

2.2 Test Cells and culture

Bhas 42 cells (v-Ha-ras-transfected Balb/c 3T3 clone A31-1-1 cells) supplied by Hatano Research Institute, Food and Drug Safety Center, Japan. Bhas-42 cells were cultured in an incubator under standard conditions (5±1% CO_2 at 37.0±1.0ºC with ≥ 85% humidity). The subculturing of the cells was performed at approximately 70% confluence of cell growth. The cells are expanded and cryopreserved in Minimum Essential Medium with 10% fetal bovine serum and 1% penicillin/streptomycin (M10F).

2.3 Cellular Transformation assay procedure

The assays was performed following the practice described by OECD guidance document (2016)[2]. The timelines for the initiator and promotor assays are shown in Figures 1 and 2 respectively. The positive controls for the initiator and promoter assays were 3-methylcholanthrene (MCA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) respectively.

Figure 1: Timeline for the initiation assay section of the CTA from OECD 2016[2]

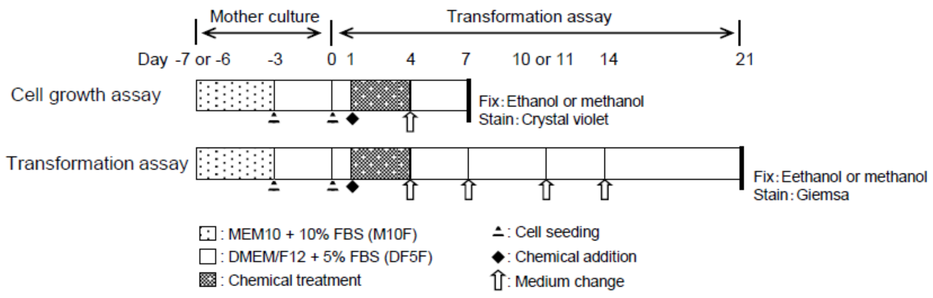
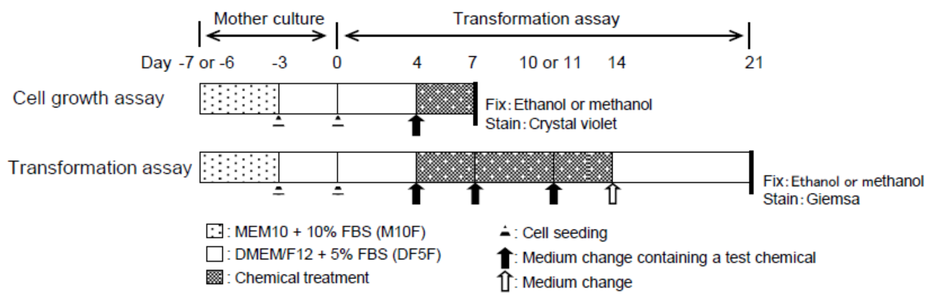


Figure 2: Timeline for the promotion assay section of the CTA from OECD 2016[2]



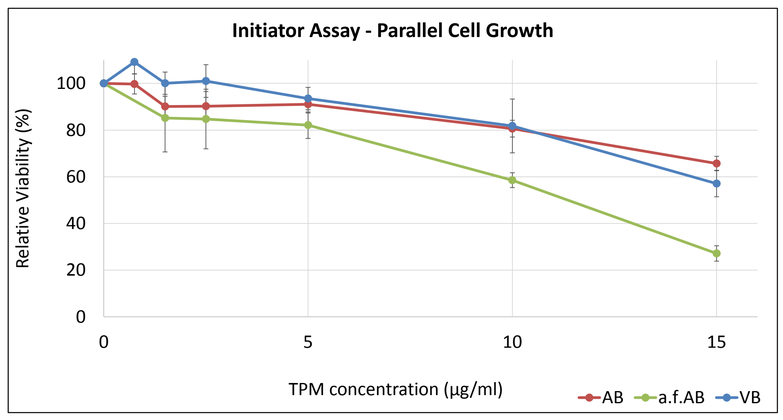
Transformed foci were analysed based on the presence of the following morphological characteristics:

- > 100 cells per foci
- Spindle-shaped cells different from the contact-inhibited monolayer cells
- Deep basophilic staining
- Random orientation of cells at the edge of foci (crisscrossing)
- Dense multi-layering of cells (piling up)
- Invasive growth into the monolayer of surrounding contact-inhibited cells

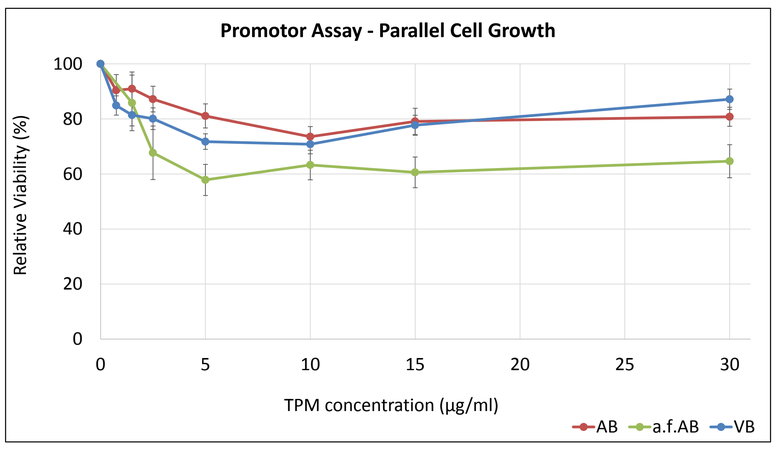
3. Results

3.1 Cell Growth Assay

Parallel cell growth assays were performed alongside the Initiator and Promotor assays.



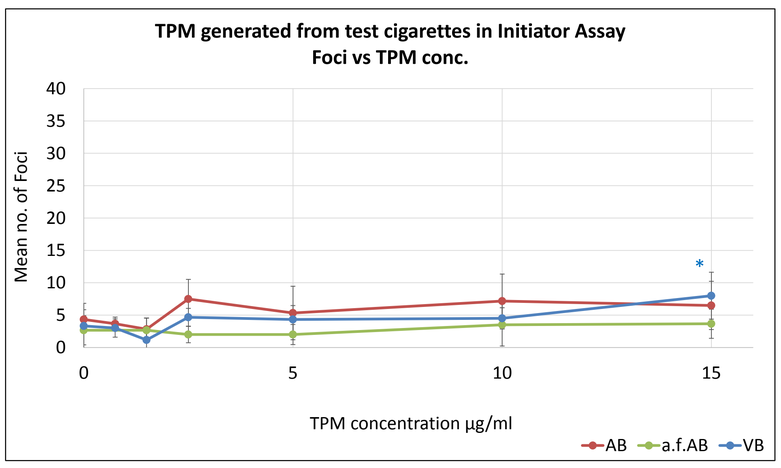
At increasing concentrations of TPM, marked decreases in cell viability were observed in the initiator assay for all three tobacco products. This was especially evident for Additive Free American Blended cigarette TPM.



Again increasing concentrations of the different TPMs caused decreases in cell viability. However, cells appeared to be more resilient to the higher concentrations of TPM than in the initiator assay.

3.2 Initiator Assay activity

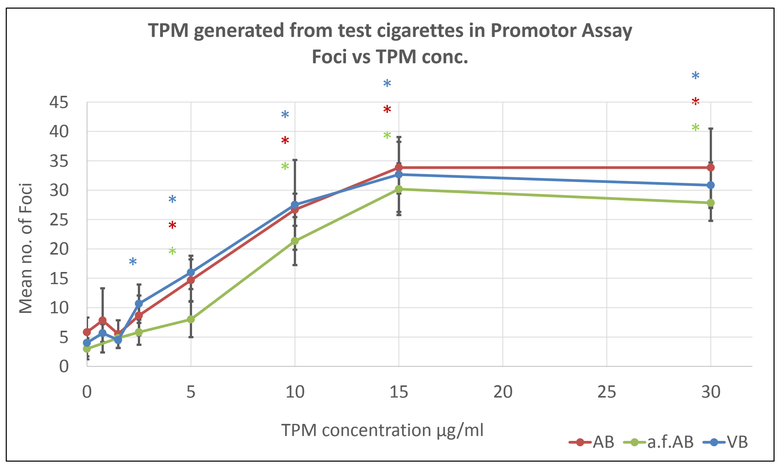
Only the TPM from the Virginia blended cigarette displayed weak initiating activity at the highest concentration tested (15 µg/ml).



* p<0.05 (ANOVA, Dunnett's post-hoc); statistically significant relative to the vehicle control

3.3 Promotor Assay activity

All three TPMs displayed Promoting activity in a concentration dependent manner. Statistically significant increases in the mean number of foci started from 2.5 µg/ml (for VB only) and 5 µg/ml (for AB and a.f.AB) relative to vehicle control.



* p<0.05 (ANOVA, Dunnett's post-hoc); statistically significant relative to the vehicle control

4. Conclusions

- Out of the different TPMs, only the Virginia blended cigarette sample displayed weak initiating activity at the maximum concentration tested (15 µg/ml), resulting in a high cytotoxicity of >40%.
- For the promotor assay statistically significant increases in the mean number of foci were seen for all different TPM at concentrations ranging from 2.5 – 30 µg/ml.
- For each Tobacco product a clear concentration response was observed in the promotor assay; highlighting the potential of this assay for future product ingredient assessment strategies or regulatory needs.

References

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