Assessment of the Genotoxicity of Tobacco Heating Products Relative to Cigarette Smoke Using the *In Vitro* Micronucleus Assay

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Introduction

This study focuses on a commercial THP (THP1.0) developed by British American Tobacco, named glo compared to Kentucky reference cigarette (3R4F). glo has been extensively investigated through a variety of chemical and analytical assessments, a battery on *in vitro* toxicological assessments, next-generation sequencing and a series of clinical studies.

In summary, glo was found to have significant reductions in chemistries/emissions. Many of the chemicals and toxicants found in cigarette smoke were not present in glo aerosol or were found to be significantly reduced[1]. As a result, glo was found to have a significantly lower impact (>98%) on Indoor Air Quality compared to conventional cigarettes[2]. In a series of *in vitro* toxicological assessments, glo was found to have lower toxicological activity when compared to cigarette smoke at equivalent doses[3-5]. RNA-sequencing based transcriptomics showed a reduced impact of glo aerosol on differential expression in MucilAir compared to cigarette smoke[6]. A biomarker clinical study demonstrated significantly reduced levels of toxicants and biomarkers of exposure in glo users' urine compared to that of regular smokers[7].

This study assesses glo TPM using the *in vitro* micronucleus assay (IVMN) with several different cell types and techniques. In the first instance V79 and TK6 cells were assessed using continuous and pulsed protocols (short +/- S9 and long -S9) and standard manual scoring methods. Secondly, a high-throughput high content screening based IVMN method was investigated using CHO-K1 cells under continuous short +S9 and long -S9 treatment conditions.

Materials and Methods

Code	Product type and manufacturer
3R4F	**Cigarette:** 3R4F scientific reference cigarette, University of Kentucky a. Tobacco b. Filter c. Paper
THP	**Tobacco heating product (THP):** Glo and Kent Neosticks (Bright Tobacco) British American Tobacco a. Tobacco b. Heat source c. Mouthpieces d. Electronics and battery e. Device body

Figure 1: Schematic representation of the products used in the study.

3R4F Kentucky reference cigarette (University of Kentucky USA)

glo (THP) commercially available tobacco heating product, comprising of device body, heating element, battery and specifically designed tobacco rod.

TPM was generated in a comparable manner for each product to a maximum final stock concentration of 50 mg/mL (1% DMSO). TPMs were stored in single use aliquots at -80°C. Reference 3R4F cigarettes and THP consumables were puffed on a Borgwaldt RM200A and a Borgwaldt LM20X linear machine (Borgwaldt-KC, Hamburg, Germany) respectively. Health Canada Intense (HCI) smoking regime (55mL puff volume, 2 sec puff duration and 30 sec puff interval, 100% vent blocking)[8] and HCI modified (no vent blocking) were used for 3R4F and THPs respectively.

Table 1: Summary of IVMN assay parameters used for biological assessment

Cells	Metabolic activation	Time points assessed	Scoring
V79 Chinese hamster lung	±S9	3 h +/- S9 24 h - S9	Manual (validated professional scorer)
TK6 Human lymphoblastoid	±S9	3 h +/- S9 24 h -S9	Manual (validated professional scorer)
CHO-K1 Chinese Hamster Ovary	±S9	3 h + S9 24 h - S9	Automated Cellomics ArrayScan® VTI HCS reader

V79, TK6 and CHO-K1 cells were supplied by the European Collection of Cell Cultures (ECACC), Salisbury, UK.

Results

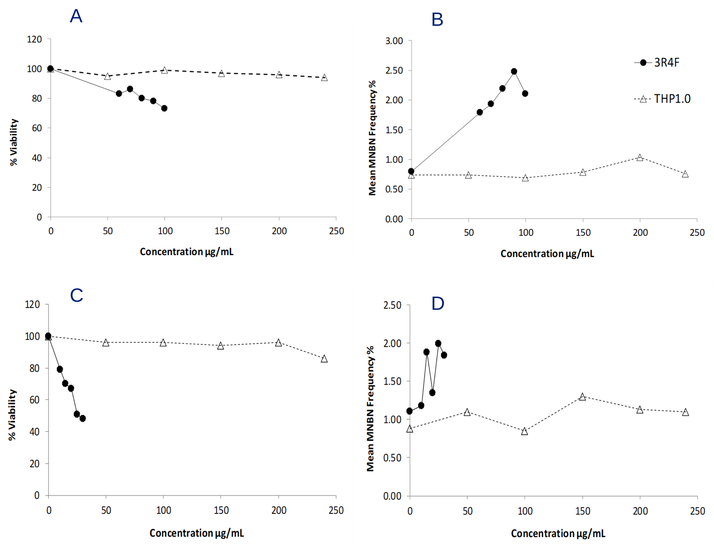

Figure 2: V79, Comparison of MNBN and viability responses for all test articles at 3 h + S9 and at 24 h -S9. A) cell viability under +S9 conditions; B) mean IVMN frequency under +S9 conditions; C) cell viability under –S9 conditions and D) mean IVMN frequency under –S9 conditions. A positive response was observed for cigarette smoke under all conditions. glo was deemed negative under all conditions

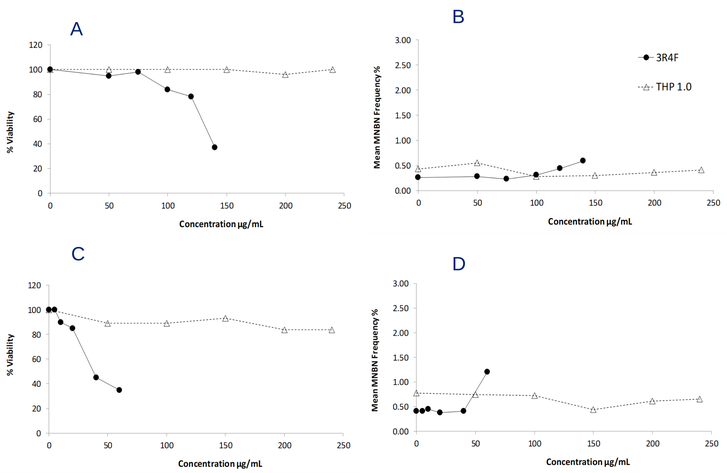

Figure 3: TK6, Comparison of MNBN and viability responses for all test articles at 3 h + S9 and at 24 h -S9. A) cell viability under +S9 conditions; B) mean IVMN frequency under +S9 conditions; C) cell viability under –S9 conditions and D) mean IVMN frequency under –S9 conditions. Cigarette smoke was deemed weakly positive under 3 h +S9 conditions and negative at 24h –S9 conditions. glo was deemed negative under all conditions

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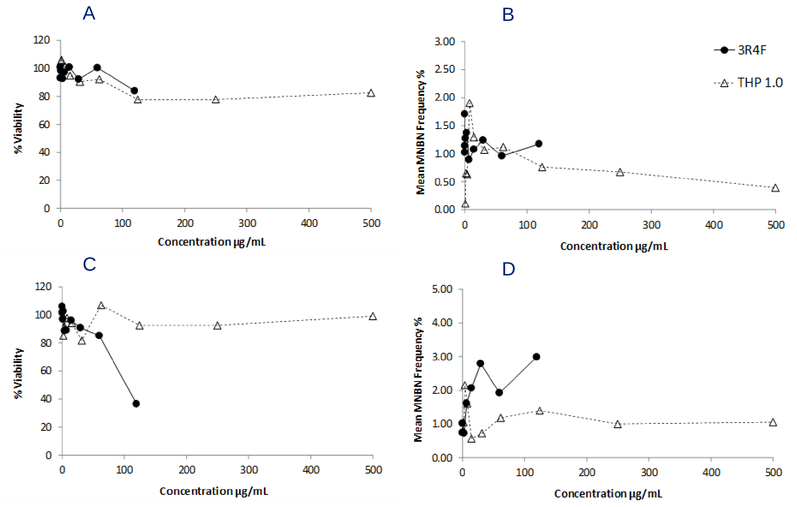

Figure 4: CHO-K1, Comparison of MNBN and viability responses for all test articles at 3 h + S9 and at 24 h -S9 using an increased THP test article concentration up to 500µg/ml. Viability calculated based on cell index in HCS screening . A) cell viability under +S9 conditions; B) mean IVMN frequency under +S9 conditions; C) cell viability under –S9 conditions and D) mean IVMN frequency under –S9 conditions. Cigarette smoke was deemed positive after 24hrs treatment –S9 and negative at 3hrs +S9. glo was negative under all conditions tested.

Conclusions

- The response to 3R4F reference cigarette smoke varied between cells types, V79s were positive under both conditions, TK6, showed a weak response and were negative with -S9 conditions and CHO were negative under +S9 conditions and positive under –S9 conditions.
- All three cell lines equally demonstrated a negative response with glo and did not increase the micronuclei formation above control levels, even at doses far exceeding that of 3R4F cigarette smoke TPM, up to 500 µg/ml.
- High content screening approaches provide a quick and valuable resource for the assessment TPM test articles and could prove useful screening tool for future studies.
- At comparable doses to cigarette smoke, glo showed no activity at all under any test condition,
- This study further supports the growing consensus that THPs are potentially less risky than conventional cigarettes

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Abstract

In vitro studies have supported the toxicological evaluation of chemicals and complex mixtures including cigarette smoke and next generation tobacco and nicotine products (NGP) which include electronic-cigarettes and tobacco heating products (THP). The NGP environment is fast-paced requiring higher throughput and more advanced *in vitro* studies, to meet the growing requirement for product innovation and the duty of care assessments. These products are believed to be less risky compared to conventional cigarette smoking, but more work is required to understand this new and continually evolving category.

In this study, total particulate matter (TPM) from two commercially available THPs (termed THP1.0 and THS) and a Kentucky reference (3R4F) cigarette were assessed using the in vitro micronucleus assay under various conditions and cell types (V79, TK6 and CHO-K1). V79 and TK6 cells were assessed under short +/- S9 and long +/- S9 conditions using standard manual scoring techniques. CHO-K1 cells were assessed under short +S9 conditions and long -S9 conditions using automated cell imagine high content screening approaches (Cellomics ArrayScan® VTI).

The response to reference cigarette smoke varied between cells types. V79 cells gave the most consistent response with all three treatment conditions producing a clear positive response. Human TK6 cells only produced a weak-positive response under one condition (3hr+S9) and CHO-K1 cells produced a positive response under long -S9 conditions, with automated scoring. However, all three cell lines equally demonstrated the same negative response with THPs up to 500 µg/ml. In many cases toxicity of the test article indicated that treatment conditions could be pushed even further. In conclusion, the THPs assessed did not increase the micronuclei formation above control levels at TPM doses far exceeding that of cigarette smoke and up to 500 µg/ml. Cigarette smoke responses were observed in the 0-70 µg/ml range depending of cell type used.

This study further supports the growing consensus that THPs are potentially less risky than conventional cigarettes and that innovative screening technologies like HCS can be employed for NGP product assessment. This will become especially important where increasing product innovations require higher throughput duty of care *in vitro* assessments.

Key Words

In vitro micronucleus assay; IVMN; Tobacco heating product; THP; TPM; TK6; V79; CHO