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The genotoxicological assessment of a tobacco heating product relative to cigarette smoke using the *in vitro* micronucleus assay

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ABSTRACT

In vitro studies have supported the toxicological evaluation of chemicals and complex mixtures including cigarette smoke and novel tobacco and nicotine products which include tobacco heating products (THP). This new environment requires faster testing, higher throughput and appropriate *in vitro* studies, to support product innovation and development.

In this study, total particulate matter (TPM) from a commercially available THP and a reference cigarette (3R4F) were assessed up to 500 μg/mL using two *in vitro* micronucleus techniques. V79 and TK6 cells were assessed using conventional OECD 487 manual scoring techniques, whereas, CHO cells were assessed using contemporary, automated high content screening approaches (Cellomics ArrayScan® VTI).

V79 cells gave the most consistent response with all three treatment conditions producing a clear positive genotoxic response. Human TK6 cells only produced dose-dependent response, indicative of a weak-positive response. CHO cells demonstrated a positive response with TPM using long (24 h) -S9 conditions. All three cell lines equally demonstrated a negative response with THP TPM up to 500 μg/mL.

In conclusion, THP TPM did not increase micronuclei formation above control levels even at doses far exceeding that tested with reference cigarette smoke, in most cases up to 10x the dose delivered compared to that of cigarette smoke. This study supports the growing belief that THPs are less risky than conventional cigarettes and that 21st century screening techniques can be employed to support product design and decision making, as a potential 1st screen prior to more traditional assessments.

1. Introduction

In the last ten years, the acceptability of tobacco alternatives, such as tobacco heating products (THP) and electronic cigarettes (e-cigarette) have increased. Innovation within the category has also increased as consumer use and insight drives product diversity and change. These products differ in their design compared to conventional cigarettes as their aerosols are generated by a process of heating (THP) and vaporisation (e-cigarette) rather than combustion. With conventional cigarettes, smoke generated by combustion and pyrolysis at temperatures exceeding 900 °C [1,2] and results in the production of thousands of chemicals and hundreds of toxicants that have been linked with various disease states [3–6]. Therefore, removing the combustion and pyrolysis process, results in a category of products that are lower in chemical and toxicant yields [7–9], although their potential to reduce health risks within a global population are still being widely debated [10].

In general, THPs utilise a tobacco rod specifically designed to work with a partner heating device. The heating device consists of a battery and microprocessor and a heating system that heats the tobacco rod up to approximately 200−400 °C. Although tobacco plant material is used

Abbreviations: 3R4F, Research reference cigarette; CRM, 81 CORESTA recommended method 81; DMSO, dimethyl sulphoxide; E-cigarette, electronic cigarette; HCI, Health Canada Intense smoking regimen; HCIm, Health Canada Intense modified smoking regimen; IVMN, *in vitro* micronucleus assay; ISO, International Standards Organisation; NGP, Next generation products; THP, tobacco heating product; TPM, total particulate matter; S9, mammalian liver post-mitochondrial fraction.

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in the tobacco rod consumable to create an aerosol similar to cigarette smoke, the actual aerosol is generated without combustion or pyrolysis. As a result, the physio-and chemical properties of a THP aerosol are significantly simpler than conventional cigarette smoke [11–14]. Recently, we have investigated a commercially available THP, glo™ (termed THP1.0) across a number of non-clinical assessments and a clinical study, all reporting the reduction in responses and biological activity when compared to a reference cigarette (3R4F) [12.15–18,14, 19–23]. The non-clinical assessments comprised of classical genotoxicity studies combined with contemporary 21st century toxicological assessments. This recent paradigm shift in toxicological assessment, is focused on using human-based cell or tissue systems with high throughput screening technologies, where multiple endpoints are simultaneously collected and analysed, aimed at reducing animal-based experimentation. The National Research Council in 2007 outlined approaches in "Toxicity testing in the 21 st century: A vision and strategy" [24] regarding advances in molecular biology, *in vitro* computational sciences to help evaluate consumer health risks and safety assessments. Such screening approaches have been employed on a large scale, for example the US Environmental Protection Agency (EPA) have used screening assays to prioritise hundreds of chemicals, through its ToxCast or "Toxicity Forecaster" programme. Using computational approaches to pull the data together the EPA is building decision-making supporting tools to not only prioritise, but to help develop predictive modelling for a number of health outcomes [25]. With the need for faster assessments not only to support product development and innovation, more modern and higher throughput *in vitro* toxicological assessments are required, and the NRC's strategy [24] has offered a natural solution to the growing complexity in the tobacco alternatives space.

These contemporary methods have also been coupled with classical testing strategies, for a combined weight of evidence approach. For example, classical genotoxicity techniques have been adapted in line with NRC's 21st century toxicity testing (TT21C) strategy, for higher throughput and aerosol exposures [26]; cytotoxicity methods have been adapted for NGPs aerosols [17,27] and contemporary high content and high throughput screening methods have been employed on both e-liquid and particulate test matrices [18,28,29]. The assessment of cellular perturbations in a systems biology approach have also been employed [21,30,31,11,18].

Classical regulatory genetic toxicology approaches such as the *in vitro* micronucleus (IVMN) assay have been employed for assessment of THPs and cigarette smoke [11,13,14]. OECD genetic toxicity test guidelines exists, for chemical assessment and have been adopted for tobacco testing [32–35]. The IVMN for example is ideal for the detection of micronuclei which may originate through a variety of mechanisms. Consequently, it provides an excellent basis for the investigation of chromosome damaging potential *in vitro* with both aneugens (agents inducing whole chromosome loss via impact to the cell mitotic apparatus) and clastogens (agents inducing chromosome fragmentation via direct DNA interaction or non-direct interference with DNA replication mechanisms). The IVMN assay has also undergone extensive validation trials [36–39] resulting in an OECD test guideline [35]. Classical approaches such as the IVMN have been extensively employed for the assessment of cigarette smoke producing clear positive responses using reference cigarettes with doses up to 240 μg/mL of total particulate matter (TPM) test material. In contrast, THPs have been shown to be negative at equivalent doses (240 μg/mL TPM) compared to cigarette smoke TPM [19]. Subsequent studies have increased test article concentration up to 1500 μg/mL with equivocal results. From 500−1000 μg/mL, no significant increase in micronuclei formation with a THP has been observed when compared to cigarette smoke [13,40]. Crooks et al., however, demonstrated an increase in micronuclei induction in response to THP test article concentrations up to 1500 μg/mL [14]. These studies demonstrate that it is possible and relevant to apply classical approaches to THP test articles but given the diversity of the environment and product development, alternative and quicker approaches need to be considered, that still capture the relevant information. For example, alternative IVMN techniques, that automate the scoring whilst increasing efficiency, potential sensitivity and throughput could be beneficial in an improved TT21C testing strategy. At the very least these approaches could prove invaluable in screening developments to evaluate whether these product changes are likely to increase consumer risk when compared to the base product.

In this study we report on the data generated from comparing classical IVMN approaches, with a contemporary approach, assessing TPM generated from a commercially available THP (glo™ (THP1.0)) to a scientific reference cigarette (3R4F). TPM preparations were assessed using the V79 s and TK6 cells using manual scoring techniques. The same TPM preparations were assessed with a fluorescence-based IVMN technique using a CHO cell line and automated scoring.

2. Materials and methods

2.1. Study design

Studies were conducted to Good Laboratory Practise (V79 and TK6) or Good Research Practice (CHO). Total particulate matter (TPM) for all products were generated to the same standard under comparable conditions, and equivalent doses were selected for all products to form a comparative study design up to 24 mg/mL not exceeding 1% DMSO. An additional comparison was made using a TPM extract at 50 mg/mL which was compared to 3R4F at 24 mg/mL. Three contrasting IVMN protocols/cells types were assessed. TPM preparations were assessed using the V79 s and TK6 cells using manual scoring techniques. The same TPM preparations were assessed with a fluorescence-based IVMN technique using a CHO cell line.

2.2. Chemicals and reagents

Where specified mammalian liver post-mitochondrial fraction (S9) was used for metabolic activation obtained from Molecular Toxicology Incorporated, USA where it is prepared from male Sprague Dawley rats induced with Aroclor 1254. All other chemicals and reagents were obtained from Sigma-Aldrich, UK unless otherwise stated.

2.3. Tobacco and tobacco heating products

A scientific reference cigarette (3R4F) and a commercially available tobacco heating product (THP1.0) as described in Table 1 and Fig. 1 were assessed in the study.

2.4. Total particulate matter (TPM) generation

TPM was generated in a comparable manner for each product. 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 (55 mL puff volume, 2 s puff duration and 30 s puff interval, 100 % vent blocking [41] and HCI modified ((HCI_m) no vent blocking) were used for 3R4F and THPs respectively. Up to 150 mg of TPM was collected onto 44 mm Cambridge filter pads ((CFPs) Whatman, Maidstone, UK) that were weighed before and after smoking to determine the mass of the deposited material. Pads were extracted into dimethyl sulphoxide (DMSO) to a final stock of 24 or 50 mg/mL, resulting in test article concentrations of 240 μg/mL and 500 μg/mL not exceeding 1% DMSO.

2.5. Cell culture

For all experiments, stocks of cells preserved in liquid nitrogen were reconstituted to maintain karyotypic stability.

Table 1
Overview of product specification.

Parameter	Product	
	Combustible cigarette [1]	Tobacco heating product version 1.0 (THP1.0) [2]
Product	Scientific research reference cigarette	Commercial tobacco heating device (THP1.0)
Commercial product name	3R4F	glo™
Aerosol generation principle	Combustion, distillation and condensation [3]	Distillation and condensation
Consumable	N/A	Kent Neostick (Bright Tobacco)
Blend style	Flue-cured, Burley, oriental and reconstituted tobacco	Blended Virginia reconstitution process
Smoking time (mins)	5	4
Puff No (#)	10	8
Smoking regimen (Puff volume (ml); puff interval (sec); puff duration (sec))	HCI [4] (55,302) 100% vent blocking	HCI_m (55,302) *No vent blocking
Smoking profile	Bell	

HCI = Health Canada Intense.
HCIm = Health Canada Intense modified.
*= vent blocking not possible with tobacco heating devices.
CA = cellulose acetate.
1 = [55].
2 = [12].
3 = [2].
4 = [41].

2.6. Chinese hamster lung V79 cells

V79 cells, were maintained in tissue culture flasks containing Dulbecco's modified Eagle's medium (DMEM) including 10 % (v/v) heat inactivated foetal calf serum (FCS) and 0.52 % penicillin / streptomycin. Cells were subcultured at a low to medium density (approximately between $1-6 \times 10^5$ cells/flask) into 75 cm^2 tissue culture flasks. Cells were passaged at least once prior to treatment. On the day prior to treatment cells were removed from stock cultures using trypsin/EDTA solution, and subcultured at a density of approximately x 10^5 cells/flask into 25 cm^2 tissue culture flasks. Pre-treatment volumes were 8.9 mL and the final volume in each flask (following completion of treatment) was 10 mL. Flasks were gassed with 5 % (v/v) CO_2 in air for approximately 20 s, sealed and incubated at 37 ± 1 °C until treatment.

2.7. Human lymphoblastoid TK6 cells

TK6 cells were maintained in tissue culture flasks containing HEPES-buffered RPMI 1640 medium with GlutaMAX-1 including 10 % (v/v) heat inactivated FCS and 100 Units/mL/100 µg/mL penicillin / streptomycin at 37 ± 1 °C, 5% (v/v) CO_2 in air, in a humidified environment. Cells were subcultured regularly at low density. The measured cell cycle time of the cells used is approximately 15−17 hours. Cells were sub-cultured at low to medium density (approximately between 5×10^4 and 1×10^6 cells/mL) into 75 cm^2 vented tissue culture flasks. Cells were passaged at least once prior to treatment. On the day prior to treatment cells were sub-cultured at a density of approximately 1×10^5 cells/mL into vented culture tubes (4.45 mL per culture for 3 + 27 h treatments; 4.40 mL for 30 + 0 h treatments). The final volume of culture medium in each tube (following completion of treatment) was 5 mL. Cells were maintained at 37 ± 1 °C, 5% (v/v) CO_2 in air, in a humidified environment prior to treatment.

2.8. Chinese Hamster Ovary CHO cells

CHO-K1 (ECACC Salisbury, UK, Collection No: CCL-61) were cultured in Ham's F12-K medium (Invitrogen) 10 % foetal bovine serum, 2 mM L-glutamine, 55 U/mL penicillin and 55 µg/mL streptomycin in 75 cm^2 cell culture flasks. Cells were maintained in a humidified atmosphere with 5 % CO_2 at 37 °C. CHO cells were plated at 2500 cells/well (−S9) or 4000 cells/well (+S9) into the wells of a 96-well clear bottomed plate, in a volume of 100 µL per well and cultured 18–22 h prior to treatment.

2.9. IVMN techniques

For each study the appropriate cytotoxicity assessment was conducted alongside the IVMN endpoint. A summary of cell systems, assay parameters and treatment conditions can be found in Table 2.

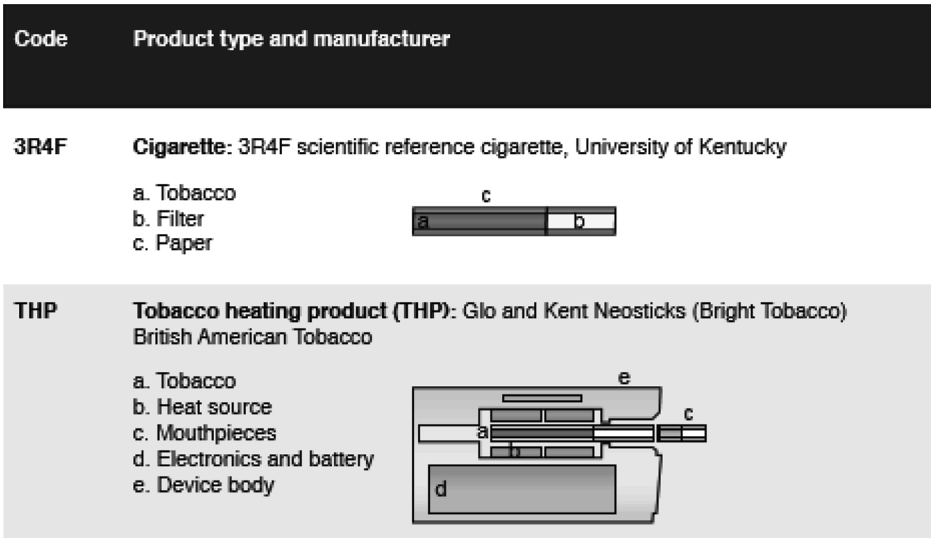

Fig. 1. Schematic representation of the products used in the study. 1) Reference cigarette (3R4F) and 2) tobacco heating product (THP1.0 T) Schematic adapted from [17].

Table 2
Summary of IVMN assay parameters used for biological assessment.

Cells	Metabolic activation	Time points assessed	Scoring
Chinese hamster lung	+/-S9	3 h +/− S9	Manual (validated competent scorer)
V79		24 h - S9	
Human lymphoblastoid	+/-S9	3 h +/− S9	Manual (validated competent scorer)
TK6		27 h -S9	
Chinese Hamster Ovary	+/-S9	3 h + S9	Automated Cellomics ArrayScan® VTI HCS reader
CH		24 h - S9	

Cells were supplied by the European Collection of Cell Cultures (ECACC), Salisbury, UK.

2.10. Manual scoring of micronuclei in V79 and TK6

All slides for micronuclei analysis were coded and scored using fluorescence microscopy under blind-scoring conditions. Prior to analysis, several drops of PBS were added to the acridine orange stained slides and the slides cover slipped. Up to 2000 binucleate cells per culture were analysed for the presence of micronuclei. Binucleate cells were only accepted for analysis if the cytoplasm remained essentially intact and the daughter nuclei were of approximately equal size. A micronucleus was recorded if it had the same staining characteristics and a similar morphology to the main nuclei, was separate in the cytoplasm or only just touching a main nucleus and was smooth-edged and smaller than approximately one third the diameter of the main nuclei. These criteria were in keeping with the principles as described by [42].

After scoring, each treatment concentration was compared with the concurrent solvent (negative) control using either the Fisher's Exact Test (Phase 1) or the Wilcoxon Rank Sum test (Phase 2) (one-sided analysis) with probability values of $p \leq 0.05$ accepted as significant [43]. A Cochran-Armitage trend test was applied to each treatment condition. Probability values of $p \leq 0.05$ were considered significant. Micronucleus frequency was also assessed against the historical solvent control (normal) range (95 % reference range, based on percentiles of the observed data).

For all tests the following acceptability criteria were fulfilled; 1) The mean frequency of cells with micronuclei in concurrent solvent controls fell within the historical solvent control (normal) ranges and 2) a minimum of 50 % of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts).

Positive control chemicals were included under each test condition and fulfilled the positive criteria as stated below. A test chemical was considered positive if the following criteria were met: 1) A statistically-significant increase in the frequency of MNBN cells at one or more concentrations was observed, and 2) The incidence of cells with micronuclei at such a concentration exceeded the historical solvent control (normal) range in both replicate cultures.

2.11. Automated Micronuclei scoring using HCS

CHO cells were assessed using fluorescent cellular imaging with an ArrayScan® VTI HCS reader (Thermo Scientific Cellomics). Two treatment conditions were assessed; 1) 3 (+21) hrs + S9 and 2) 24 h-S9. Using HCS multiple endpoints are simultaneously collected, including, relative survival (cell count), membrane integrity (cytotoxicity assessment) and cell cycle information (binucleated cell frequency and proliferation index (CBPI)). In combination these parameters are used to determine cell health (cytostasis) and micronuclei validity. Minimum effective concentration (MEC) values represent where a positive micronucleus result is observed (greater than 3-fold over vehicle control). Briefly, the IVMN assay was performed as has been described previously [44]. Etoposide (-S9) and cyclophosphamide (+S9) were used as assay controls and dosed at the appropriate concentration alongside the test samples. For the + S9 treatment, cells were treated with compounds for 3 h with a rat liver S9 (1% w/v, BioIVT) metabolic regeneration system (McCoy's 5A media, 0.8 mg/mL of NADP, 1.5 mg/mL of isocitric acid), followed by a media wash and fresh media replacement for 19−21 h. For the − S9 treatment, cells were treated with compounds continuously for 22–24 h. Following test sample exposure, the media was removed, cells were washed once, and fresh medium containing 6 μg/mL of cytochalasin B (Santa Cruz Biotechnology) was added to the cells for a period of 22–24 h to block cytokinesis. The cytokinesis block was subsequently removed, the cells washed once and then fixed by adding 100 μl of 37 °C fixing solution containing 3.7 % formaldehyde and 1 μM of Hoechst dye (Thermo Fisher Scientific) for 20 min. The cells were then washed twice with PBS and left remaining in 200 μL of PBS. The plates were sealed and scanned using a Cellomics® ArrayScanVTI High Content Screening Reader (ThermoFisher Scientific Inc., Waltham, MA) using the software vHCS™ view software (ThermoFisher Scientific Inc.), using the micronucleus bioapplication. A 20X objective was used using autofocus per each field of view. Micronuclei were scanned in binucleated cells and validate by both cell nuclei being of similar size and intensity and the micronuclei size being ≤0.33 the size of the cell nuclei and similar intensity. A total of 1000 binucleated cells per well in duplicate wells was determined per test concentration, fewer than 1000 where considered as cytotoxic. A positive result was defined as a ≥3-fold increase in the percentage of micronucleated cells compared to the corresponding vehicle control wells. A weak (+/-) positive was defined as a relative increase between ≥2-fold and <3-fold in the percentage of micronucleated cells compared to vehicle control wells.

As previously described [44] for cytotoxicity assessment, a modified version of the cytotoxicity block proliferation index (CBPI) was used. The percentage of cytotoxicity was defined as: $100 - 100(CBPIt-1)/(CBPIc-1)$, where CBPI = (number of mononucleated cells + 2×number of binucleated cells)/total number of cells; CBPIt = CBPI of treated cells; CBPIc = CBPI of control cells. In addition, a cytostasis index based on cell numbers was also calculated, in which the percentage of cytostasis was defined as: 100−(average number of cells per field (treated) ×100/average number of cells per field (Control). As such micronucleated cells were considered apoptotic if the binulcleated cells were below 30 %, cytostasis was >50 and less than 1000 binucleated cells were remaining.

3. Results

Chinese hamster V79 fibroblasts were used to assess TPM from a reference cigarette (3R4F) and a commercial THP (THP1.0). TPM preparations were assessed using manual scoring techniques at 3 h +/− S9 and at 24 h -S9. 3R4F exposure in the absence and presence of S9 resulted in frequencies of MNBN cells which were significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle controls. Positive MNBN induction was observed well within the ranges of specified toxicity [35].

Exposure with THP1.0 T TPM did not result in the significant ($p \leq 0.05$) induction of MNBN cells above control levels. Single exceptions were observed following THP1.0 exposures at 24 + 0 h treatment in the absence of S9 at a concentration of 150 μg/mL and at a concentration of 200 μg/mL following 3 + 21 h -S-9 treatment. However, the magnitude of these increases were within 95th percentile of the control ranges. For other treatments the MNBN cell frequency of all treated cultures did not differ from control cultures. Viability of THP1.0 exposures even at the top doses assessed (up to 500 μg/mL) did not fall below 90 % and therefore did not reach the required level of toxicity as described by OECD 487 (Fig. 2).

Human lymphoblastoid TK6 cells were also used to assess TPM from reference cigarette (3R4F) commercial THP (THP1.0). TPM preparations

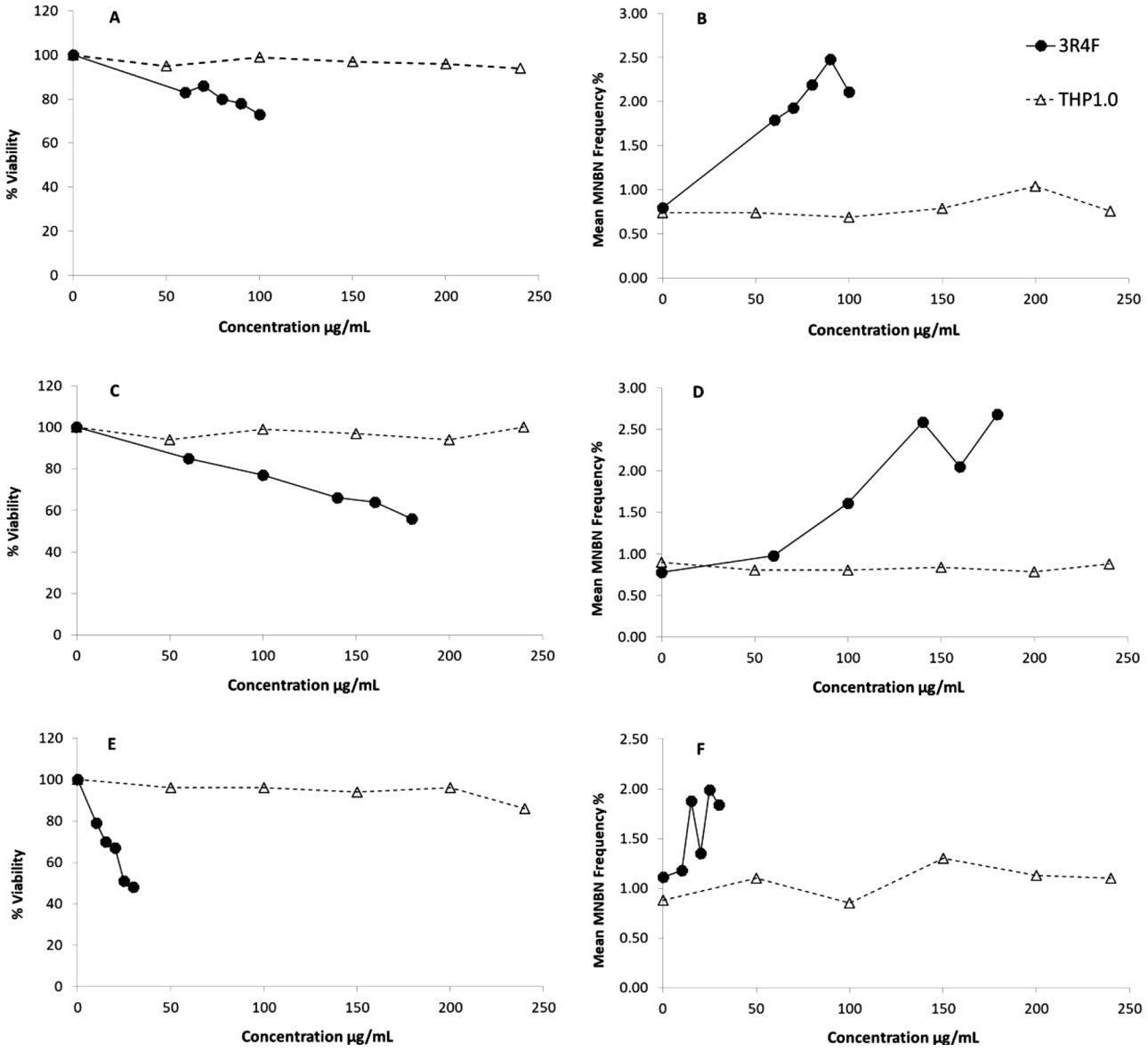

Fig. 2. *In vitro* micronucleus testing, following OECD TG TG487 using V79 and manual scoring techniques. Responses at 3 (+21) h +/− S9 and at 24 (+0) h -S9 for 3R4F and THP1.0 T. A, C and E represent viability; B, D and F represent corresponding % MNBN cell frequency. A and B represent 3 h -S9 treatment condition. C and D represent 3 h + S9 treatment condition and E and F represent 24 h -S9 treatment condition. A positive response for cigarette smoke was observed under all three treatment conditions, whereas THP1.0 T was deemed negative under all conditions.

were assessed using manual scoring techniques at 3 h (+27) +/− S9 and at 30 (+0) h -S9. Under 3 h (+27) - S9 conditions, no significantly increases ($p \leq 0.05$) in MNBN frequencies were observed and responses were comparable to those observed in control doses. At 3 h (+27) +/− S9 elevated frequencies of MNBN were observed for all five concentrations analysed with a positive linear trend. However, these increases did not exceed the normal historical ranges. Overall, these data were considered to indicate evidence of a weak response. Clear reductions in viability were observed for 3R4F treatments under all treatment conditions.

Treatment with THP1.0 in the absence and presence of S9 resulted in frequencies of MNBN cells which were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle controls for all concentrations analysed (up to 240 µg/mL). Cytotoxicity up to the OECD limit was not observed at the maximum dose assessed (240 µg/mL). The maximum concentration dosed and analysed was 240 µg/mL, which represents the maximum achievable concentration using standard extraction approaches, limited by the concentration of the solvent (DMSO) at 1% (Fig. 3).

Chinese hamster ovary CHO cells were used to assess TPM from reference cigarette (3R4F) and commercial THP (THP1.0) using a fluorescence-based technique. Using this method up to 120 µg/mL of TPM was tested, based on a maximum concentration of DMSO up to 0.5 %. Two treatment conditions were assessed, 3 h + S9 and 24 h -S9. At 3 h + S9, no response was observed with either 3R4F or THP1.0 and little evidence in toxicity were observed for any product. At 24 h -S9 a positive response was observed for 3R4F with corresponding cytotoxicity at the top dose (120 µg/mL). Conversely, THP did not produced a positive response or a reduction in cell viability even at the top dose tested (120 µg/mL) (Fig. 4).

Given the limits of DMSO an additional experiment was conducted at higher TPM concentrations. In this case, the concentration of THP1.0 TPM was increased to 600 µg/mL with a maximum level of 0.83 % DMSO (which was confirmed not to have an effect based on vehicle controls), resulting in a top dose of 500 µg/mL, Increasing THP1.0 TPM dose to 500 µg/mL had no obvious effect on toxicity or MNBN induction,

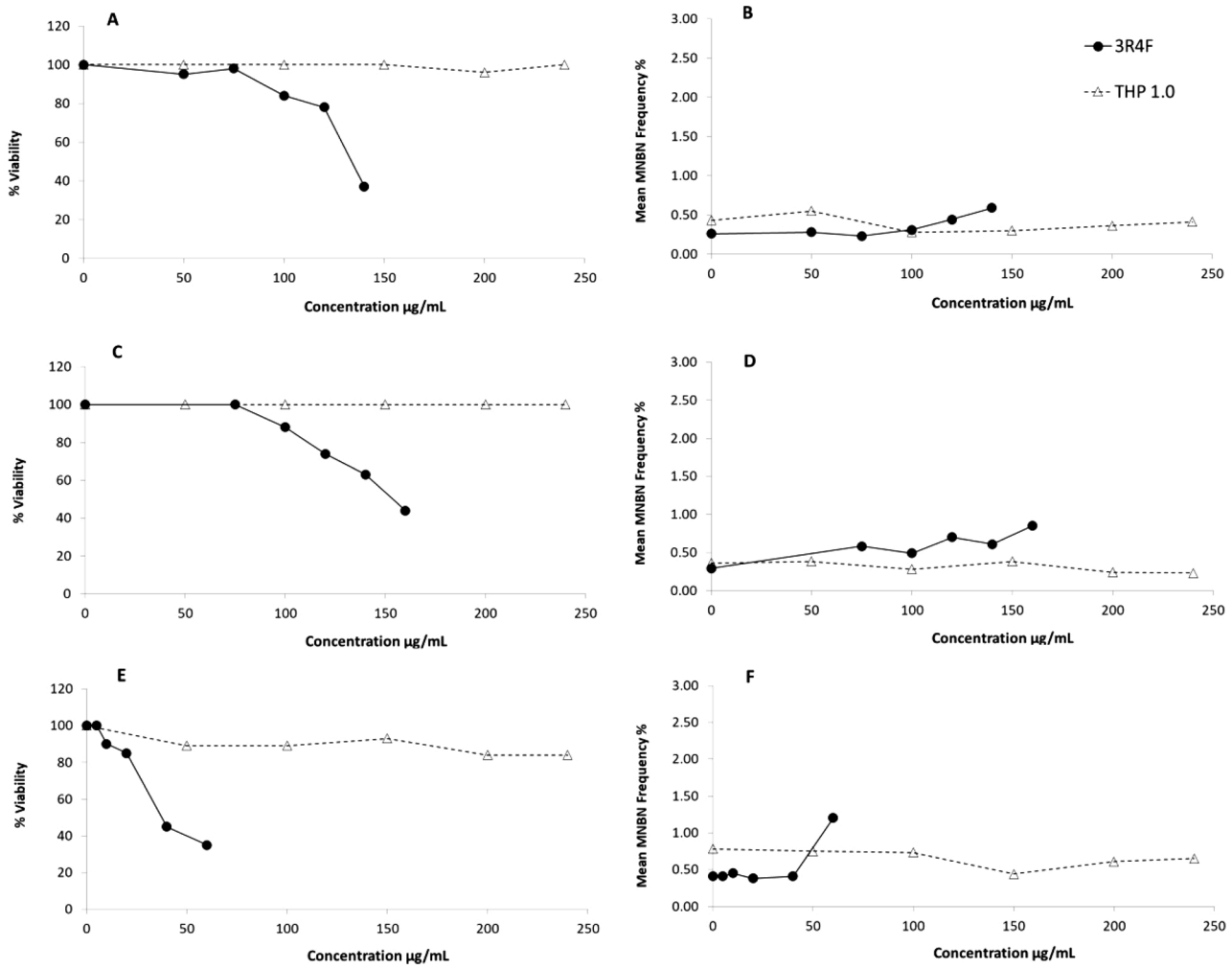

Fig. 3. *In vitro* micronucleus testing, following OECD TG TG487 using TK6 and manual scoring techniques. Responses at 3 (+27) h +/− S9 and at 30 (+0) h -S9 for 3R4F and THP1.0 T. A, C and E represent viability; B, D and F represent corresponding % MNBN cell frequency. A and B represent 3 h -S9 treatment condition. C and D represent 3 h + S9 treatment condition and E and F represent 30 h -S9 treatment condition. A weak-positive response for cigarette smoke was observed at 3 h + S9, whereas THP1.0 T was deemed negative under all conditions.

with levels not exceeding background controls at doses for exceeding that of 3R4F cigarette smoke (Fig. 5).

Table 3 shows a summary and classification of the results obtained for all IVMN protocols employed for the assessment of 3R4F and THP1.0.

4. Discussion

The aim of the study was to assess a commercialised THP (THP1.0) against reference cigarette smoke (3R4F), by using a variety of IVMN techniques and cell lines, employing classical and contemporary TT21C approaches for image/scoring and assessment. The goal was to demonstrate that TT21C screening approaches could be used to support testing strategies, due to their higher-throughput potential, which can be considered an advantage compared to traditional manual scoring techniques.

In response to 3R4F cigarette smoke exposure, the various protocols (cell lines, treatment conditions and scoring techniques) produced a mixed response. Cigarette smoke TPM produced a positive response across all three treatment conditions for V79 cells. TK6 cells showed a negative response in two out of the three treatment conditions, with only a weak response observed using the 3 h + S9 treatment condition. CHO cells, using HCS scoring techniques demonstrated a positive response in the 24 h -S9 exposure whereas the 3 h + S9 exposure failed to elicit a response. Where a positive response was observed for CHO cells, the magnitude (fold increase) was comparable to that observed for V79 cells (3.7 vs. 3.4 for CHO and V79 s respectively). TK6 cells produced a significant linear trend, but the increases observed were within control ranges (<1 % MNBN). Therefore, the response observed was only considered to be indicative of a weak-positive response at best. Meaning for the most part, TK6 cells failed to pick up a response using 3R4F cigarette smoke test articles. Interestingly, despite cell line differences, the respective toxicities of 3R4F for the various treatment conditions were all relatively equal. This shows that V79, TK6 and CHO cell lines are responding equally to the toxicity of 3R4F cigarette smoke, but not in the formation of micronuclei.

With THP1.0 TPM, irrespective of exposure condition or cell type, a negative response was observed. This was even apparent when TPM dose was increased to 500 µg/mL, no evidence of increased toxicity or increasing micronuclei formation was observed either. THP TPM exposure did not meet the required 50–60 % toxicity threshold as per OECD TG487 [35], whereas cigarette smoke TPM did in almost all treatment conditions. This data suggests that THP exposure can be tested to higher levels than the top dose of 500 µg/mL as tested here. Future studies should investigate generating a more concentrated TPM test article in order to push the *in vitro* test system to high levels of toxicity as described by OECD (OECD). Several studies on the genotoxic potential of THPs have concluded similar findings as compared to this study. In

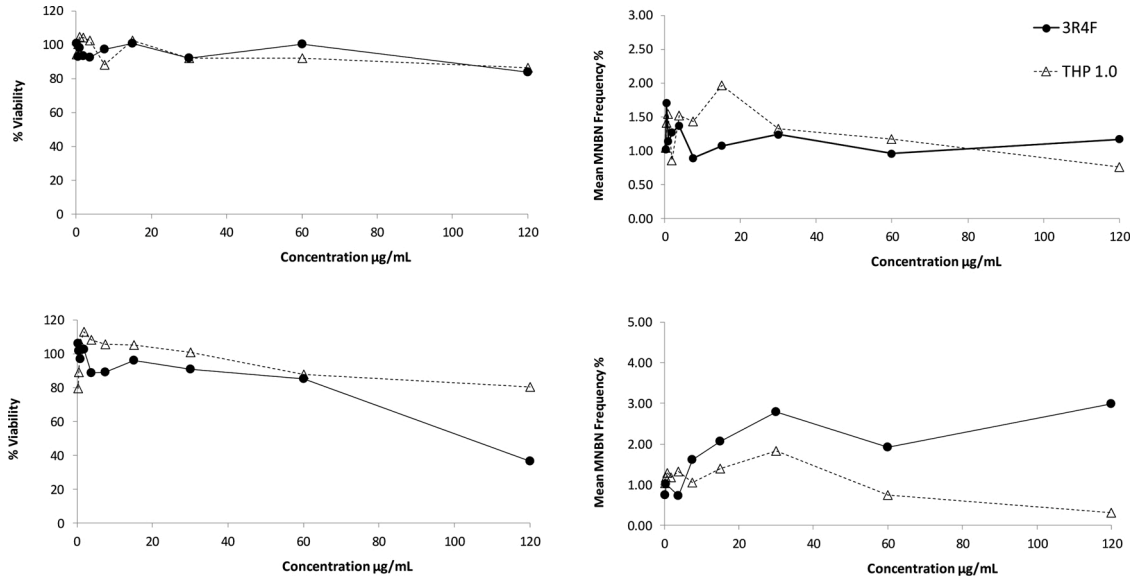

Fig. 4. *In vitro* micronucleus testing, using CHO and 21st Century automated scoring techniques (Cellomics® ArrayScanVTI High Content Screening Reader). Responses at 3 h + S9 and at 24 (+0) h -S9 for 3R4F and THP1.0 T. A and C represent viability; B and D represent corresponding % MNBN cell frequency. A and B represent 3 h + S9 treatment condition. C and D represent 24 h -S9 treatment condition. A positive response for cigarette smoke was observed at 24 h - S9, whereas THP1.0 T was deemed negative under both conditions up to 120 µg/mL.

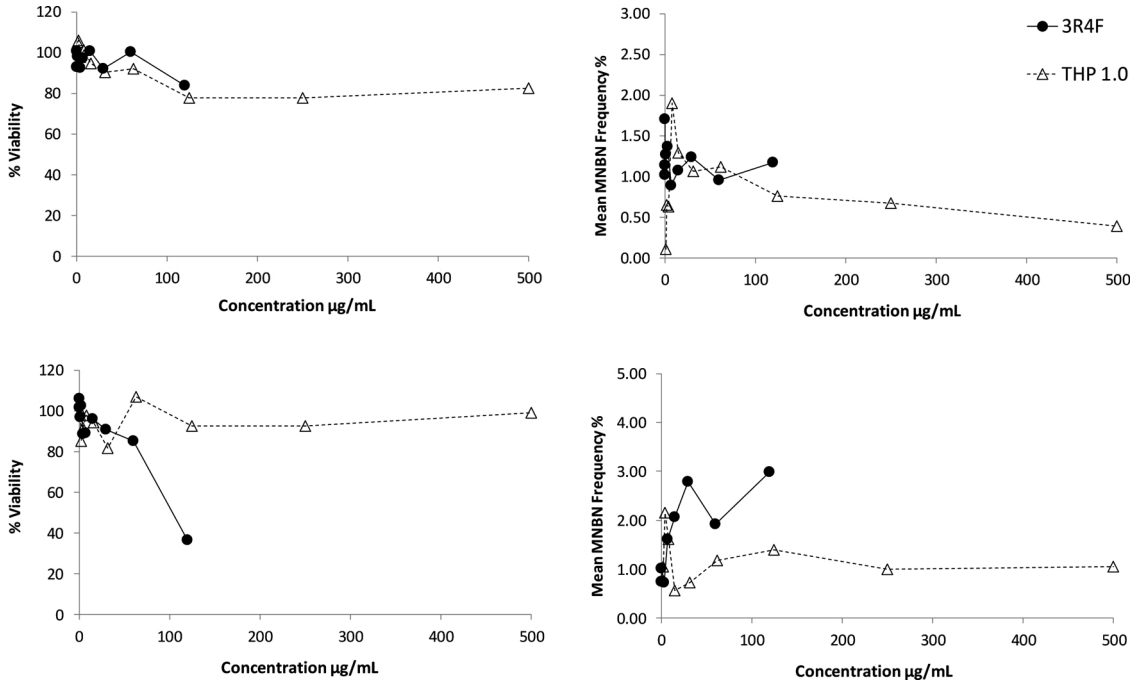

Fig. 5. *In vitro* micronucleus testing, using CHO and 21st Century automated scoring techniques (Cellomics® ArrayScanVTI High Content Screening Reader). Responses at 3 h + S9 and at 24 (+0) h -S9 for 3R4F and THP1.0 T. A and C represent viability; B and D represent corresponding % MNBN cell frequency. A and B represent 3 h + S9 treatment condition. C and D represent 24 h -S9 treatment condition. A positive response for cigarette smoke was observed at 24 h - S9, whereas THP1.0 T was deemed negative under both conditions up to 500 µg/mL.

these studies, cigarette smoke was deemed positive and THP test articles were negative at comparable concentrations as assessed in this study [11,14,19]. Few studies have tested THP TPM concentrations further than the levels assessed here. Where THP TPM concentrations have been assessed to extreme levels, 2–2.5 fold as compared to those tested in this study, mixed responses were observed. For example [13], tested a THP compared to cigarette smoke TPM and demonstrated little to no activity in cytotoxicity (up to 1000 µg/mL using the NRU assay), mutagenicity in the Ames assay (5000 µg/plate) and genotoxicity in the IVMN assay (1000 µg/mL). In contrast, cigarette smoke produced clear positive responses with comparable ranges to those observed in this study [14] also demonstrated increased dosing of a THP TPM compared to cigarette

Table 3
Summary of results.

Cell line	Treatment condition	Cigarette Smoke Classification (fold change where a positive response was observed)	Tobacco Heating Product (THP1.0)
V79 Manual Scoring	3 h + S9	+ve (3.1)	−ve
	3 h - S9	+ve (3.4)	−ve
	24 h - S9	+ve (1.9)	−ve
TK6 Manual Scoring	3 h + S9	+ve (2.5)	−ve
	3 h - S9	−ve	−ve
	30 h - S9	−ve	−ve
CHO Automated scoring	3 h + S9	−ve	−ve*
	24 h - S9	+ve (3.7)	−ve*

+ve = positive response observed.
−ve = negative response observed.
*= increased dose range up to 500 μg/mL.

smoke and observed positive responses of THP TPM at approximately 1500 μg/mL in the IVMN assay using V79 cells. This study took THP TPM 10-fold the dose, that was required to elicit a biological response in cigarette smoke (150 μg/mL for cigarette smoke and 1500 μg/mL for THP). These studies demonstrate that it is possible to achieve much higher TPM concentrations and even generate positive responses. However, such exposures and responses must be caveated. Increasing TPM concentrations may result in exceeding the OECD guidance on solvent use (not exceeding 1 %). Concentrations should also be contextualised against consumer use to understand what these increased doses represent in terms of biological exposure. Chemical characterisation should be considered with generation of higher TPM stock concentrations to ensure that the ratio of chemicals present in THP aerosols, are not adversely affected when using artificially high doses. Any response observed at high doses should be contextualised against aerosol chemistries and TPM characterisation to ensure concentrating the test article has not inadvertently caused proportional changes and selective enrichment of the TPM test article. Currently, no study which has used increased TPM concentrations has investigated whether the ratios of chemicals present are consistent compared to what would be expected from the source aerosol, or whether artificial enrichment has occurred. This could be especially important where positive responses have been observed. The overall conclusion of these studies remains and extending the dose range is clearly important, especially in achieving top doses and required levels of toxicity. However, the means and ratios in which the chemicals are delivered could be significantly different than the source THP aerosol. Therefore, the conclusions of such studies should be appropriately considered.

In a comparable study using e-cigarettes, THP and cigarette smoke, Thorne et al. [40,45] using the same test articles showed that cigarette smoke was deemed positive in both IVMN and MLA assays at comparable doses to those assessed in this study. The Next Generation Products (NGPs) e-cigarette an THP, showed no activity across both the MLA and IVMN assays. An interesting outcome for this work was the application of an extended exposure/recovery period for the IVMN assay which demonstrated increased responsiveness to cigarette smoke. Based on these findings, the authors propose that this increased recovery period could be applied to the assessment of THP in future studies to potentially increase assay sensitivity. Finally, in the same study, multiple cell types were assessed, V79, CHO and TK6 cells. The findings in this study correlate with those from [40] in that different cell types all responded in varying degrees to cigarette smoke, with TK6 and CHO cells largely unresponsive under standard conditions and V79 cells showing positive responses under all treatment conditions. It was only with the application of an extended recovery/expression period that responses in TK6 and CHO cells became evident. The addition of the extended exposure/recovery period has also been recently shown to be advantageous for increasing the IVMN assay sensitivity [46] and could help with test article discrimination within category. However, these studies did not investigate alternative contemporary approaches such as high content screening, as investigated here.

Finally, this study has compared different IVMN techniques, several cell lines and scoring methods, aimed at higher throughput to support screening approaches. Considering the current level of innovation in the e-cigarette and THP space, and technology developments, *in vitro* testing approaches need to evolve towards higher throughput approaches to meet the demands of the diverse category and evolution not only within category but across current, new and emerging categories. In part, classical approaches are time consuming and expensive to conduct and can't be, from a practicality perspective, conducted on all product variants. In this study we have investigated only one high throughput high IVMN approach using a cell fluorescence-based platform. Other high throughput IVMN approaches exist, such as flow-cytometry based techniques which have been developed in a multitude of cell lines, TK6, V79 and CHO's for example [47–49]. In these high throughput approaches 10,000 cells can be analysed in as little as 2 min vs. 600 cells in 15 min using traditional scoring approaches [48]. Furthermore, it was reported that automated scoring could reduce man hours per study by 70 %, increase data turn around by 50 % and due to the assessment of large cell numbers, the variability of the automated technique could be lowered too [50]. Due to the nature and applicability of these techniques with both suspension and attachment cell lines, such an approach could easily be combined with whole aerosol exposure methodologies, to create a modern version of the classic IVMN assay. This combination has been briefly investigated with traditional scoring approaches [51,52], but as yet it does not feature in any whole aerosol testing strategy. IVMN high throughput is not the only high throughput approaches being developed. New high throughput approaches such as the MultiFlow® flow and ToxTracker® assays are showing extremely positive results from a mechanistic and screening perspective [53,54]. Furthermore, these techniques are showing good concordance with classical genetic toxicological approaches, suggesting that they could be employed as a fast pre-screen prior to any large-scale *in vitro* study. These high throughput techniques could in the future enable the screening large numbers of compounds, which may assist and help guide the toxicological evaluation of some of these new categories such as tobacco heating products.

In conclusion, this study has demonstrated that a high throughput fluorescence-based IVMN technique can be used as part of a screening approach for THPs and that the results produced are consistent with standard methodologies in terms of fold-change. The advantage of such screening methods is that they use less materials, both cellular and test material, often the data analysis is automated or at least semi-automated, enabling quicker analysis and interpretation of the results, and ultimately this will increase time to a go/no-go or additional work required decision. This approach can support the generation of early data on tobacco heating products and other novel categories, which in turn will help streamline and focus the required battery of *in vitro* tests required to support regulatory decision making.

Authors statement

All authors jointly designed the study. DT wrote the manuscript with support from all authors. All authors approved the final version.

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Declaration of Competing Interest

David Thorne, Damien Breheny and Marianna Gaca are employees of British American Tobacco (BAT). Glo is manufactured by BAT. Julie Clements and James Whitwell are employees of Covance laboratories.

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