Food and Chemical Toxicology 132 (2019) 110546

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Genotoxicity evaluation of tobacco and nicotine delivery products: Part Two. *In vitro* micronucleus assay

David Thorne[a,*], Robert Leverette[b], Damien Breheny[a], Mel Lloyd[c], Stephen McEnaney[c], James Whitwell[c], Julie Clements[c], Betsy Bombick[b], Marianna Gaça[a]

[a] British American Tobacco, R&D, Southampton, Hampshire, SO15 8TL, UK
[b] RAI Services Company, 401 N. Main Street, Winston-Salem, NC, 27101, USA
[c] Covance Laboratories Ltd., Otley Road, Harrogate, North Yorkshire, HG3 1PY, UK

ARTICLE INFO

Keywords:
In vitro
NGP
THP
E-cigarette
IVMN
TPM
E-liquid
Nicotine equivalents

ABSTRACT

In this study, a variety of test matrices from tobacco and nicotine delivery products were assessed against a 3R4F Kentucky reference cigarette using the *in vitro* micronucleus assay. Testing was conducted using two Chinese hamster cell lines (CHO and V79), and a human lymphoblastoid cell line (TK6), in accordance with established guidelines.

Total particulate matter (TPM) from a 3R4F Reference cigarette was compared to an electronic cigarette e-liquid, electronic cigarette TPM and TPM from a commercial tobacco heating product using a standard and an extended treatment condition with recovery period.

Cells were assessed with 3R4F TPM prior to assessment of the other tobacco and nicotine product test matrices. These cell lines gave varied responses to 3R4F TPM with the most robust response using V79 cells. The use of an extended exposure/recovery period was seen to increase assay sensitivity for CHO and V79 cell lines but was less clear for TK6 cells.

Negative responses were observed for all products except 3R4F across all treatment conditions in V79 cells. The most potent response to cigarette smoke was following extended treatment with recovery, suggesting this may be a more appropriate treatment for the future assessment of tobacco and nicotine product test matrices.

1. Introduction

Next generation tobacco and nicotine delivery products (NGPs) comprised of tobacco heating products (THP) and electronic-cigarettes (e-cigarettes) have evolved significantly over the last few years and are gaining consumer acceptability. Current opinion holds that NGPs may present less risk to health compared to conventional tobacco smoking (McNeill et al., 2015). Several researchers have reported a significant reduction in chemicals and toxicants yielded by THPs and e-cigarettes relative to conventional cigarettes (Farsalinos and Le Houezec, 2015; Forster et al., 2018; McNeill et al., 2015; Margham et al., 2016; Schaller et al., 2016; Smith et al., 2016). Additionally, *in vitro* and *in vivo* clinical data suggest that these products have significantly lower biological activity compared to cigarette smoke (Ay and Kacker, 2014; Farsalinos and Polosa, 2014; Gale et al., 2018; Haswell et al., 2018; Hecht et al., 2015; Jaunky et al., 2018; Misra et al., 2014; Schaller et al., 2016).

There is currently no international consensus on what pre-clinical *in vitro* tests are most appropriate for initial screening of the toxicological potential of NGPs. As part of a pre-clinical assessment strategy, a battery of *in vitro* tests could be used for the initial screening of the toxicological potential of NGPs. Previous studies have reported using various *in vitro* techniques for screening purposes, which include (but are not limited to): classical genotoxicity techniques, cytotoxicity, contemporary high content and high throughput screening methods and the assessment of cellular perturbations, coupled to adverse outcome pathways in a systems biology approach (Clippinger et al., 2018; Haswell et al., 2018; Murphy et al., 2018; Schaller et al., 2016; Taylor et al., 2018).

For more classical approaches, clear international guidelines exist, which recommend various *in vitro* mutagenicity and genotoxicity assays. These include guidelines developed by the International Conference on Harmonisation (ICH S2(R1) guideline) (2011) for pharmaceuticals, the UK Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (2011), and, guidance for combustible cigarettes from Health Canada (2005) and the Cooperation Centre for Scientific Research Relative to Tobacco

* Corresponding author.
E-mail address: David_Thorne@BAT.com (D. Thorne).

https://doi.org/10.1016/j.fct.2019.05.054
Received 28 February 2019; Received in revised form 6 May 2019; Accepted 29 May 2019
Available online 01 June 2019

(CORESTA) (2004). These guidelines collectively call for the use of the bacterial mutagenicity assay (Ames reverse mutation assay), the *in vitro* micronucleus assay (IVMN) and/or the *in vitro* mouse lymphoma assay (MLA), combined with an acute cytotoxicity assay. Depending on guidelines, the chromosome aberration assay can also be used (CORESTA, 2004; ICH, 2011).

Most known or suspected human genotoxic carcinogens have been demonstrated to be mutagenic in animal or human somatic cells *in vitro*. Current knowledge indicates that the endpoints most relevant to the induction of cancer and heritable genetic damage are point mutations, structural chromosome aberrations and chromosome loss events (aneuploidy). The IVMN assay is a genotoxicity test for the detection of micronuclei within the cytoplasm of interphase cells. Micronuclei may originate from acentric chromosome fragments (that lack a centromere), or whole chromosomes that are unable to attach to the mitotic spindle and are left behind within the cell cytoplasm as chromosomes move during mitosis. Consequently, the IVMN test 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 e.g. via direct DNA interaction or non-direct e.g. interference with DNA replication mechanisms) in cells that have undergone cell division during or after exposure to a test chemical. As such, this single *in vitro* assay covers two of the three important endpoints for assessment of genotoxicity and following extensive validation trials (Aardema et al., 2006; Clare et al., 2006; Kalweit et al., 1999; Kirsch-Volders, 1997; Kirsch-Volders et al., 2003; Lorge et al., 2006; Migliore and Nieri, 1991; Miller et al., 1998; Oliver et al., 2006; Wakata et al., 2006) was endorsed for regulatory genotoxicity testing with the adoption of Organisation for Economic Co-operation and Development (OECD) Test Guideline 487 (TG487) in 2010, updated in 2014 and 2016 (OECD, 2016). This assay has also been recommended for assessing the relative toxicities of tobacco smoke and tobacco smoke condensates (CORESTA, 2004).

The IVMN assay was further adapted with the development of the cytokinesis block method with cytochalasin B (Albertini and Kirsch-Volders, 1997; Fenech and Morley, 1986). Cytochalasin B inhibits cytokinesis (cell division) but not karyokinesis (nuclear division), resulting in the formation of binucleate cells. Using this technique, when micronuclei are counted in binucleate cells, a true reflection of micronuclei can be obtained (Fenech, 2000; OECD, 2016). This method is mandated for use in human lymphocytes (where mixed cell populations are present) and optional for immortalised cell lines (OECD, 2016).

In this study, the IVMN (cytokinesis block) assay was used for the assessment of NGPs. THP and e-cigarette particulate-based test articles were compared to cigarette smoke total particulate matter (TPM). E-cigarette liquid (e-liquid) was also compared to TPMs from cigarette smoke, THP and an e-cigarette. Finally, NGP test articles were compared to cigarette smoke based on a nicotine equivalence to demonstrate consistent dosing across test articles. This manuscript describes the use of cigarette smoke in the IVMN assay to establish optimum assay parameters (cell type and recovery period) and secondly, for the assessment of NGPs. This manuscript is Part Two of a two-part series where the same test articles and TPM preparations were compared in both the MLA and the IVMN (detailed here) assay, in a coordinated testing approach (Thorne et al., 2018b).

2. Materials and methods

All testing was conducted in general concordance with OECD TG487 (OECD, 2016). All chemicals and reagents were obtained from Sigma-Aldrich UK, unless otherwise stated.

2.1. Study design

The aim of the study was to identify optimal assay parameters (cell lines and extended recovery periods) for the assessment of cigarette smoke and NGPs using the *in vitro* micronucleus assay. The study was broken down into two distinct phases. Phase 1 established optimised conditions and compared three different cells lines (rodent CHO, V79 and human lymphoblastoid TK6) on their ability to respond to cigarette smoke TPM with the inclusion of an extended treatment/recovery period (either with or without a 1.5–2 cell cycle recovery period). An extended recovery period was included for the long-term exposure since the OECD 487 guidelines state that the use of an extended recovery phase may be necessary for test articles that do or are suspected to affect cell cycling time, and more so for p53 competent cell lines (OECD, 2016). Phase 2 was designed to use these optimised conditions (cell type and treatment/recovery period/cigarette smoke TPM concentrations) to assess NGP test articles in comparison to cigarette smoke. To achieve this two-phased approach, TPM from a combustible cigarette, a THP and an e-cigarette were generated and tested alongside the same e-liquid used to generate the e-cigarette TPM.

2.2. Test articles

Cigarette, THP and e-cigarette TPM, and e-liquid were assessed in this study. These were evaluated in the form of four separate test articles. TPM was collected from 3R4F reference cigarette (University of Kentucky, USA), THP and e-cigarette aerosols, while the e-liquid from the same e-cigarette was also assessed directly, to determine if the aerosolization process imparted any differential genotoxicity on the e-liquid in comparison to that from the e-liquid itself.

3R4F Kentucky Reference cigarette TPM was generated on a RM200 smoking machine (Borgwaldt, Hamburg, Germany) according to the Health Canada Intense (HCI) smoking regimen, described as a 55 mL puff every 30 s with a 2 s puff duration and 100% vent blocking (Health Canada, 1999), bell shaped puff profile. THP TPM was generated on a RM200a smoking machine using a Modified HCI puffing regime, described as a 55 mL puff volume every 30 s with a 2 s puff duration, but with no vent blocking. E-cigarette TPM was generated on a RM200a smoking machine using the CORESTA recommended method 81 puffing regime described as a 55 mL puff volume every 30 s with a 3 s puff duration, square wave puff profile to allow product actuation via puffing (CORESTA, 2015).

For 3R4F cigarettes, THPs and e-cigarettes, 10, 12 and 60 puffs were used per consumable/cartomiser, respectively. Fresh consumables/cartomizers and fully charged devices (where applicable) were used on every occasion to achieve maximum and consistent aerosol delivery, and ultimately collection for extraction and assessment purposes. Separate smoking machines were used for cigarette smoking as compared to THP and e-cigarette TPM collections to avoid cross-product contamination. In a recent study by Adamson et al. (2017), the authors demonstrated that two distinct exposure systems can perform the same if appropriately maintained. Therefore, the use of separate smoke generation systems to prepare TPM from different products was preferable to avoid cross-category-contamination.

TPM from cigarettes and NGPs was collected onto 44 mm Cambridge filter pads (Whatman, Maidstone, UK). Pads were weighed before and after TPM collection to determine the mass of the deposited material, before being extracted in anhydrous analytical grade dimethyl sulphoxide (DMSO), to a final stock concentration of 20 mg/mL for cigarette smoke and 50 mg/mL for NGPs. TPMs were stored in single use aliquots at −80 °C. E-liquid was diluted/prepared in DMSO prior to use. DMSO was use as a vehicle control throughout the study.

For all products, duplicate CFP pads (termed partner pads) were smoked/puffed on each day of TPM generation. In addition to these pads, a quality control (QC) pad was also collected every 10th pad smoked. These duplicate and QC pads were analysed for nicotine, water and glycerol content using GC-TCD and GC-FID techniques. The rationale for conducting duplicate analysis on separate pads (partner pads), is to give a TPM, nicotine, water and humectant measure, to increase

pad replicates and to ensure there is enough material for biological analysis. These measures can be used for control purposes to assess the quality of the TPM preparations. Partner and QC pads were analysed for nicotine, water and glycerol content using GC-TCD and GC-FID techniques. TPM characterisation can be found in Thorne et al. (2018b) (Part One), as the same TPMs were employed across studies.

2.3. Metabolic activation system

Mammalian liver post-mitochondrial fraction (S9) prepared from male Sprague Dawley rats induced with Aroclor 1254 was used for metabolic activation (Molecular Toxicology Incorporated, USA). The S9 was supplied as lyophilized S9 mix (Mutazyme™, MolTox, USA), stored frozen at < -50 °C, and thawed and reconstituted with purified water to provide a 10% S9 mix prior to use.

Treatments were carried out both in the absence and presence of S9 by addition of either 150 mM potassium chloride or 10% S9 mix respectively to a final volume of 1% (v/v) in the test system.

2.4. Cell culture

All cell lines used in this study were tested and found to be negative for mycoplasma contamination. Cell proliferation times, expressed as average generation time (AGT) (Palma et al., 1993), were calculated from sham-treated cultures by use of bromodeoxyuridine incorporation assay. In order to maintain cell characteristics, in particular karyotypic stability, all cell cultures were reconstituted from stocks preserved in liquid nitrogen for each experiment performed.

2.5. CHO-WBL cells

CHO cells, supplied by the European Collection of Cell Cultures (ECACC), were maintained in tissue culture flasks containing McCoy's 5 A medium (Gibco, Paisley, UK) supplemented with 10% (v/v) heat inactivated foetal calf serum (HIFCS; Life Technologies, UK) and 52 Units/mL penicillin/52 μg/mL streptomycin (PAA laboratories, Leeds, UK). The modal chromosome number for the clone of CHO cells used at the testing laboratory was 21. The measured cell cycle time of the cells used is approximately 12–13 h. Cell sheets were removed from stock cultures using trypsin/EDTA solution and subcultured at a low to medium density (approximately between 5-6 x 10^5 cells per flask) into 75 cm^2 tissue culture flasks. Cells were passaged at least once prior to treatment. On the day prior to treatment, cell sheets were removed from stock cultures using trypsin/EDTA solution and subcultured at a density of approximately 6-7 x 10^5 cells per flask into 25 cm^2 tissue culture flasks. For extended treatment with recovery (24 + 24 h -S9), cultures were established at approximately 3-5 x 10^5 cells per flask into 25 cm^2 tissue culture flasks. The final volume in each flask (following completion of treatment) was 10 mL. Flasks were gassed with 5% (v/v) CO_2 in air, sealed and incubated at 37 ± 1 °C until treatment. Cultures at a suitable level of confluence were selected for treatment (40–50% or 25–35% confluency for the 3 h and 24 h treatments, respectively).

2.6. V79 cells

V79 cells were obtained from the European Collection of Cell Cultures (ECCAC, Salisbury, UK) and were maintained in tissue-culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with supplemented with 10% HIFCS and 52 Units/mL penicillin/52 μg/mL streptomycin (PAA laboratories, Leeds, UK). The modal chromosome number for the clone of V79 cells used at the testing laboratory was 22. The measured cell cycle time of the cells used was approximately 9–12 h. Cell sheets were removed from stock cultures using trypsin/EDTA solution and subcultured at a low to medium density (approximately between 1-6 x 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 5-7 x 10^5 cells/flask into 25 cm^2 tissue culture flasks. For extended treatment with recovery (24 + 24 h -S9), V79 cultures were established at approximately 3-5 x 10^5 cells/flask into 25 cm^2 tissue culture flasks. The final volume in each flask (following completion of treatment) was 10 mL. Flasks were gassed with 5% (v/v) CO2 in air, sealed and incubated at 37 ± 1 °C until treatment. Cultures at a suitable level of confluence were selected for treatment (40–50% or 25–35% confluency for the 3 h and 24 h treatments, respectively).

2.7. TK6 cells

TK6 cells were obtained from the European Collection of Cell Cultures (ECCAC, Salisbury, UK) and were maintained in tissue culture flasks containing RPMI 1640 medium with GlutaMAX™ (Life Technologies, UK) and supplemented with 10% HIFCS and 52 Units/mL penicillin/52 μg/mL streptomycin (PAA laboratories UK). TK6 cells were found to have an average generation time of approximately 15–17 h and a modal chromosome number of 47. Cells were subcultured 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 (every 2–3 days as appropriate). On the day prior to treatment, cells were subcultured at a density of approximately 1×10^5 cells/mL into 10 mL or 15 mL vented culture tubes. 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. All cultures were incubated on a slope.

2.8. Selecting doses for micronucleus analysis

Range-finding experiments were performed to determine a suitable range of TPM and e-liquid concentrations for testing. Multiple concentrations of each product were tested to enable micronucleus analysis on approximately equitoxic concentrations, where applicable, and to fulfil the following criteria: 1) highest concentration: inducing approximately 50–60% cytotoxicity; 2) intermediate concentration: inducing approximately 20–40% cytotoxicity, and 3) lowest concentration: inducing less than 20% cytotoxicity.

In the absence of cytotoxicity, samples were tested to the maximum concentration possible, limited as a result of product formulation and the necessity to hold DMSO concentration to 1% (v/v), per OECD guidelines for the IVMN assay (OECD, 2016).

2.9. Treatment

Exponentially growing cultures of all cell types were treated with the solvent or multiple concentrations of each test article or positive control chemical, under the conditions shown in Tables 1 and 2.

The final culture volume was either 5 mL (TK6) or 10 mL (CHO, V79) at the time of treatment. The cell cultures were incubated at 37 ± 1 °C for the designated exposure time. After the desired treatment times, test products were removed, and cells washed twice either by rinsing flasks containing cells growing in mono-layer (CHO, V79) or by centrifugation (TK6). Cultures were re-fed with fresh medium containing 10% HIFCS and Cytochalasin B at a final concentration of 3 μg/mL for all cell cultures. For the extended treatments without recovery, Cytochalasin B was added to all treatment cultures (3 μg/mL) at the start of treatment and remained until the time of cell harvest.

2.10. Harvesting

Following treatment and recovery, cells were harvested by either removing the monolayer (CHO, V79) with trypsin/EDTA or by centrifugation to collect the cells (TK6).

Table 1
Treatment conditions.

Phase 1 – Assay Optimisation						
Cell line	Test Article	Treatment + recovery (h)	S9	Removal oftest/positive control chemical (h post treatment)	Addition ofCytochalasin B (h)	Harvest time (h)
TK6	3R4F TPM (200 μg/mL)	3 + 27	+/−	3	3	30
		30 + 0	–	30	0	30
		27 + 27	–	27	27	54
CHO/V79		3 + 21	+/−	3	3	24
		24 + 0	–	24	0	24
		24 + 24	–	24	24	48
Phase 2 – NGP assessment						
V79	3R4F TPM (200 μg/mL)	3 + 21	+/−	3	3	24
	THP TPM (500 μg/mL)	24 + 24	–	24	24	48
	E-cigarette TPM (500 μg/mL)					
	E-liquid (500 μg/mL)					

Table 2
Culture replicate conditions.

Phase 1 - Assay Optimisation					
Toxicity Range Finder	Treatment (hours)	Cell Line	Number of cultures Vehicle	Test Article	Positive Control
	3 + 21, -S9	CHO/V79	2	1	0
	3 + 21, +S9		2	1	0
	24 + 0, –S9		2	1	0
	24 + 24, -S9		2	1	0
	3 + 27, -S9	TK6	2	1	0
	3 + 27, +S9		2	1	0
	30 + 0, –S9		2	1	0
	27 + 27, -S9		2	1	0
Micronucleus Experiment	24 + 0, -S9	CHO/V79	4	2	2
	24 + 24, -S9		4	2	2
	3 + 21, + and -S9		4	2	2
	30 + 0, -S9	TK6	4	2	2
	27 + 27, -S9		4	2	2
	3 + 27, + and -S9		4	2	2
Phase 2 – NGP assessment					
Micronucleus Experiment	24 + 24, -S9	V79	6	4	4
	3 + 21, +S9		6	4	4
	3 + 21, -S9		6	4	4

At the specified sampling time (Table 1), cells from each treatment culture were sampled for measurement of cytotoxicity and micronuclei. After the above sampling had occurred, cells from each culture for micronuclei analysis were collected by centrifugation and treated with a hypotonic solution of 75 mM KCl at 37 °C for 4 (TK6) or 5 (CHO, V79) minutes to swell the cells to aid microscopic analysis. Cells were fixed with methanol:acetic acid (7:1) fixative using several washings and centrifugation steps. Slides were prepared and stained with a solution of 12.5 μg/mL acridine orange (Sigma) and stored in the dark (typically for no longer than 1 week) until slide analysis.

2.11. Assessment of cytotoxicity

Slides were examined, uncoded, for cytotoxicity to a minimum of either 200 (toxicity range-finder assessments), or 500 cells per culture. From these data, the Replication Index (RI) which indicates the relative number of cell cycles per cell during the period of exposure of Cyto-B, was determined to calculate cytotoxicity (OECD 487), defined as shown in Fig. 1.

A suitable range of concentrations was selected for micronucleus analysis based on toxicity data in line with current regulatory guidelines for the *in vitro* micronucleus assay (OECD, 487).

2.12. Scoring of micronuclei

All slides for micronucleus 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 nucleus, was separate in the cytoplasm or only just touching a nucleus and was smooth-edged and smaller than approximately one third the diameter of the nucleus. These criteria were in keeping with the principles as described by Fenech et al. (2003). For Phase 1, the same analyst scored all slides from all treatments. For Phase 2, treatments were split between different analysts (though the same analyst scored all replicates per concentration from any one treatment condition).

2.13. Data analysis

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 $p < 0.05$ accepted as significant (Richardson et al., 1989). A Cochran-Armitage trend test was applied to each treatment condition. Probability values of $p < 0.05$ 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 (Hayashi et al., 2011).

For all tests, the following acceptability criteria were fulfilled: 1) The mean frequency of cells with micronuclei in concurrent solvent controls fell within the laboratory's 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. At the time of this study,

$$\text{Cytotoxicity (\%) is expressed as 100 relative RI} = \frac{(\text{No. binucleated cells}) + (2 \times \text{No. multinucleate cells}))}{\text{Total number of cells treated cultures}} \times 100$$

Fig. 1. Calculation for % cytotoxicity expressed as RI.

historical solvent control data was not available for the extended treatment with the added recovery period; therefore, the historical solvent control data for the extended treatment without recovery was used for the analysis.

A test chemical was considered negative in this study if none of the above criteria were met.

Results which only partially satisfied the above criteria (i.e. being neither clearly positive nor clearly negative) were assessed by expert judgement to conclude on the likely biological relevance, e.g. where positive results were only observed at cytotoxic concentrations (greater than 50% cytotoxicity). Evidence of a concentration-related effect was considered useful but not essential in the evaluation of a positive result.

3. Results

All raw data can be found in supplementary material, broken down by experiment with statistical observations (Tables S1–S6).

3.1. Phase 1 – optimisation of protocols

Three cell lines (CHO, TK6 and V79) were assessed using 3R4F reference cigarette smoke TPM with the inclusion of an extended recovery period. Four treatment conditions were assessed as indicated in Tables 1 and 2.

Following cigarette smoke TPM treatment, CHO cells did not show a positive induction of MNBN cells above that of concurrent controls in either the 3 h (-S9), 3 h (+S9) or 24 h (-S9) treatment condition. The MNBN cell frequency within these treatment conditions and doses fell within normal historical ranges for vehicle control. Following extended 24 h treatment with 24 h recovery, statistically significant increases in MNBN cells were observed with a general concentration-related response apparent (significant linear trend). Both treated cultures at the high concentration of 140 μg/mL exhibited MNBN cell values that exceeded historical vehicle control data (generated for 24 + 0 h treatments) (Fig. 2).

Human lymphoblastoid (TK6) cells did not show a clear positive response to 3R4F TPM under any treatment condition, including the extended recovery period (27 + 27 h -S9). MNBN frequencies were similar to and not significantly ($p < 0.05$) higher than those observed in concurrent vehicle controls for the majority of concentrations analysed. Some exceptions were observed. For example, at the highest concentration analysed post 27 + 27 h treatment, a statistically significant increase in MNBN cells was observed. However, the magnitude of increase was small and comparable to a low concurrent vehicle control response. The MNBN cell frequency of both test article treated cultures at this concentration (and all other concentrations) fell within normal laboratory historical vehicle control ranges. Following 3 + 27 h (-S9) treatment, the highest concentration tested and analysed (200 μg/mL) only induced 12% cytotoxicity, while the recommended maximum cytotoxicity level is 50–60% in OECD TG487 (OECD, 2016).

Pulse (3 + 27 h) treatment in the presence of S9 resulted in significantly ($p \leq 0.01$) elevated frequencies of MNBN cells for all four concentrations analysed with a positive linear trend apparent. However, these increases were not large, with just single cultures at concentrations of 150 and 175 μg/mL exhibiting MNBN cell values that exceeded normal ranges. The MNBN cell frequencies of all other 3R4F cigarette TPM treated cultures fell within normal laboratory historical vehicle control values. Therefore, data were considered to indicate evidence of a weak test article related effect (Fig. 3).

Statistically significant increases in MNBN cells were observed following 3R4F smoke TPM treatment in V79 cells under the following conditions: 3 + 21 h (-S9) and in the extended 24 + 24 h (-S9) treatments. The MNBN cell frequencies of both replicate cultures at the two highest concentrations analysed (both treatment arms) exceeded historical vehicle control data, with a concentration-related response apparent. In contrast, no such increases in MNBN cells were observed following continuous 24 + 0 h (-S9) treatment for all concentrations analysed. Following 3 + 21 h (+S9) treatment, a small but statistically significant increase in MNBN cell frequency was observed at a single intermediate concentration. However, this increase was not large, with MNBN cell values exceeding the normal range for just one of the two treated cultures. Single cultures at lower and higher concentrations analysed also marginally exceeded the normal range. These data were considered to indicate weak induction of MNBN cells (Fig. 4).

Upon assessment of positive controls, all three cells lines responded to positive controls, demonstrating the validity of the test system for each cell line (Table 3).

In summary of Phase 1 results, protocol optimisation of the three cell lines was assessed with cigarette smoke TPM and all three cell lines produced a positive response under at least one treatment condition. V79 cells appeared to be the more sensitive cell line to tobacco smoke constituents, with three of the four treatment conditions triggering a positive or weak-positive response. TK6 cells produced two weak-positive responses out of the four treatment conditions, while CHO cells produced one clear positive response from the four treatment conditions. With the exception of TK6 cells, the extended recovery period

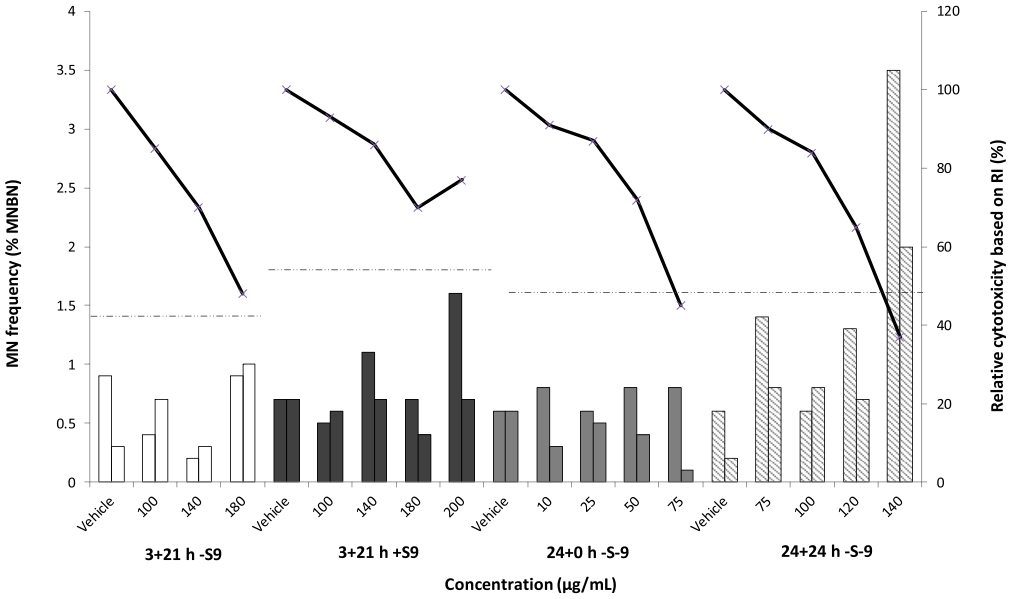

Fig. 2. 3R4F smoke TPM: comparison of IVMN response in CHO cells. MN induction and relative RI following 3 h treatment with and without metabolic activation (S9) and recovery or following 24 h treatment with extended 24 h recovery. Bar charts show MN frequency of individual replicate cultures. Line graphs show relative cytotoxicity calculated based on RI. MN: micronucleus; MNBN: micronucleated binucleate cells; RI: replication index; dashed line = upper level of historical vehicle MNBN control range.

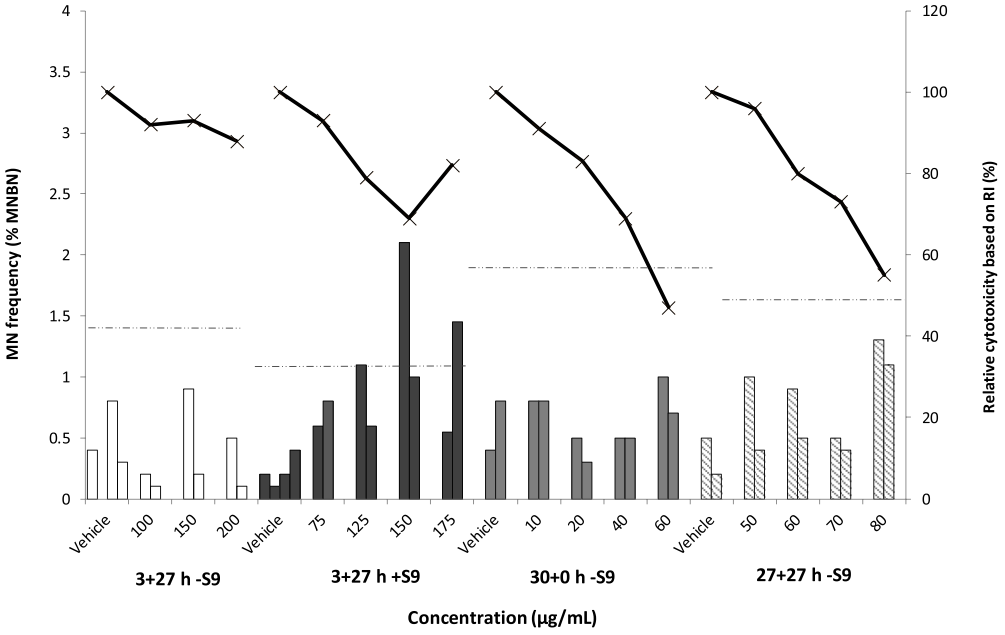

Fig. 3. 3R4F smoke TPM: comparison of micronucleus response in human TK6 cells. MN induction and relative RI following 3 h treatment with and without metabolic activation (S9) and 27 h recovery or following 27 h treatment with 27 h recovery. Bar charts show MN frequency of individual replicate cultures. Line graphs show relative cytotoxicity calculated based on RI. MN: micronucleus; MNBN: micronucleated binucleate cells; RI: replication index; dashed line = upper level of historical vehicle MNBN control range.

notably increased responses in both V79 and CHO cells. However, the increased response in CHO cells was accompanied by > 60% cytotoxicity. As an outcome from Phase 1, V79 cells were selected for NGP assessment. Finally, the 24 + 0 h (-S9) treatment condition was shown to be negative in each cell type to cigarette smoke TPM and was dropped for Phase 2, in favour of including the extended treatment with recovery (24 + 24 h -S9).

Table 4 gives a breakdown of the treatment conditions and observed responses for each of the cell types.

3.2. Phase 2 – NGP assessment

Cigarette smoke (TPM), THP (TPM) and e-cigarette (TPM and e-liquid) were assessed using V79 cells. The extended treatment with recovery (24 + 24 h -S9) period was utilised, while the 24 + 0 h (-S9) treatment condition was removed, for the reasons identified in Phase 1, in that the test condition was consistently unresponsive in all cell types and therefore deemed superfluous.

Clear positive statistical responses were observed for cigarette smoke TPM under the three treatment conditions (3 h ± S9 and 24 + 24 h -S9). Similarly, to Phase 1, the extended treatment with recovery period facilitated a greater magnitude of response (measured MNBN cells) in response to cigarette smoke. Neither the THP nor e-cigarette (TPM or e-liquid) samples produced a positive response under any test condition (including the extended treatment with recovery despite exceeding the TPM dose equivalent for 3R4F exposures) (Fig. 5).

TPM-based approaches typically display the resulting response data as a function of particulate weight (TPM µg/mL). However, this may not always be fully appropriate or sufficient when assessing NGPs, where the aerosols are chemically and compositionally very different.

Table 5 shows the summary of induced MNBN frequencies for cigarette, THP and e-cigarette (TPM and e-liquid), presented as a function of nicotine concentration within the test article, as calculated from the stock solutions. The data show that on all occasions at the top dose of cigarette smoke TPM and where a positive response was observed, NGPs (THP and e-cigarette) gave clear negative responses at nicotine dose levels approximating and exceeding those seen for cigarette smoke TPM exposures.

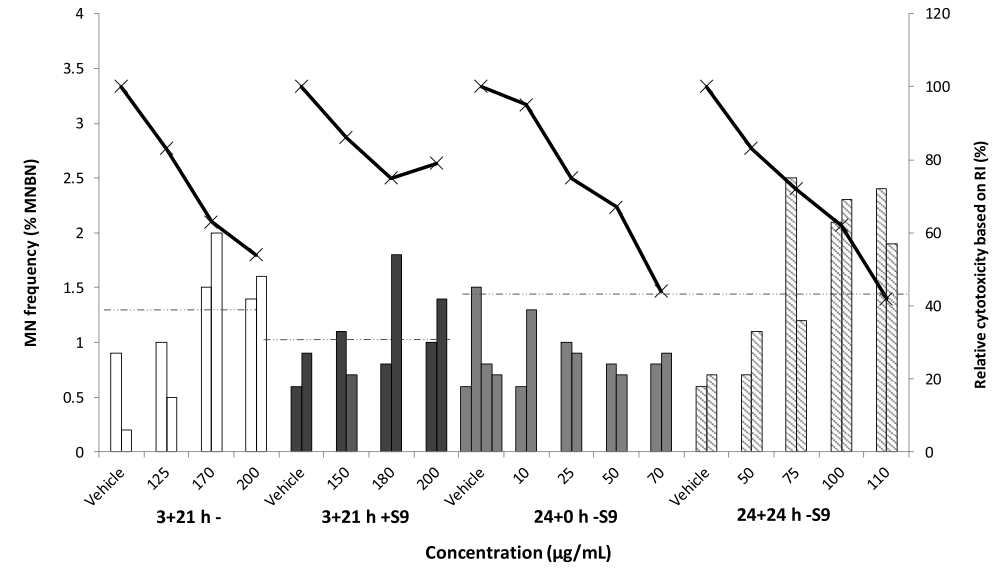

Fig. 4. 3R4F smoke TPM: comparison of IVMN response in V79 cells. MN induction and relative RI following 3 h treatment with and without metabolic activation (S-) and 17 h recovery or following 24 h treatment with 24 h recovery. Bar charts show MN frequency of individual replicate cultures. Line graphs show relative cytotoxicity calculated based on RI. MN: micronucleus; MNBN: micronucleated binucleate cells; RI: replication index; dashed line = upper level of historical vehicle MNBN control range.

Table 3
Summary of positive control results.

Treatment Conditions	Cells	Control concentration (μg/mL)	Cytotoxicity (%)	Mean MNBN frequency (%)
3 + 21 h -S9	CHO	Vehicle	~	0.60
	CHO	MMC 0.20	17	6.90
	V79	Vehicle	~	0.55
	V79	MMC 0.20	10	5.15
3 + 27 h -S9	TK6	Vehicle	~	0.38
	TK6	MMC 0.20	42	3.05
3 + 21 h +S9	CHO	Vehicle	~	0.70
	CHO	CPA 4.0	36	7.20
	V79	Vehicle	~	0.75
	V79	CPA 3.0	50	4.05
3 + 27 h +S9	TK6	Vehicle	~	0.23
	TK6	CPA 3.0	47	1.95
24 + 0 h -S9	CHO	Vehicle	~	0.60
	CHO	NOS 20.0	53	16.80
	V79	Vehicle	~	0.90
	V79	NOS 30.0	8	24.35
30 + 0 h -S9	TK6	Vehicle	~	0.60
	TK6	NOS 20.0	24	5.05
24 + 24 h -S9	CHO	Vehicle	~	0.40
	CHO	NOS 7.50	0	0.90
	V79	Vehicle	~	0.65
	V79	NOS 20.0	57	3.85
27 + 27 h -S9	TK6	Vehicle	~	0.35
	TK6	NOS 10.0	52	2.05

MMC = Mitomycin C; CPA = Cyclophosphamide; NOS = Noscapine.
NB. All positive control treatments caused a significant induction in MNBN ($p < 0.05$).

Table 4
Summary of responses to 3R4F TPM and increased recovery period.

Condition	3 + 21 h (-S9)[b] 3 + 27 h (-S9)[c]	3 + 21 h (+S9)[b] 3 + 27 h (+S9)[c]	24 + 0 h (-S9)[b] 30 + 0 h (-S9)[c]	24 + 24 h (-S9)[b] 27 + 27 h (-S9)[c]
CHO	Negative[a]	Negative[a]	Negative	Positive[a]
V79	Positive	Weak positive[a]	Negative	Positive
TK6	Negative[a]	Weak positive[a]	Negative	Weak positive[a]

[a] = Did not reach 50–60% cytotoxicity as per OECD Test Guideline 487.
[b] = CHO & V79 treatment schedules.
[c] = TK6 treatment schedules.

4. Discussion

The aim of the study was to identify optimal assay parameters (cell lines and exposure/extended recovery periods) for the assessment of cigarette smoke and NGPs, using the IVMN assay. The study was broken down into two distinct phases. Phase 1 was designed to establish optimised conditions and compared three different cells lines (CHO, TK6 and V79) on their ability to respond to reference cigarette (3R4F) smoke TPM. In addition, an extended treatment including a recovery period of 1.5–2 cell cycles was assessed. Phase 2 was designed to use the optimised conditions (cell type and treatment conditions) to assess NGPs against cigarette smoke. In order to achieve this two-phased approach, TPM from cigarette smoke, THP and an e-cigarette were collected and tested alongside the same e-liquid that was used to generate the e-cigarette TPM.

The results from Phase 1 demonstrated that in all cell lines assessed (V79, CHO, TK6), cigarette smoke TPM induced micronuclei following treatment under at least one test condition, either (or both) in the absence or presence of S9. For both CHO and V79 cells, the most potent response was observed following the extended treatment with recovery period (24 + 24 h -S9). For TK6 cells, increased frequencies of MNBN cells were observed following treatment in the presence of S9 at subtoxic concentrations. The CHO cells gave a positive response only after the inclusion of the recovery period after extended treatment. These data contrast with other studies that have demonstrated positive responses using CHO cells and cigarette smoke TPM. For example, Takahashi et al. (2018) showed positive responses in CHO cells under all treatment conditions (3 h ± S9 and 24 h -S9). These differences in responses may be due to variation in specific cell batches or laboratory methodologies. In summary, V79 cells were considered the most responsive to cigarette smoke constituents especially following the 24 + 24 h (-S9) treatment. The increased response observed from the extended recovery/expression period, suggests that the compounds in cigarette smoke causing MNBN, may also be responsible for causing a delay in cell cycle, such that inclusion of a recovery period following extended exposure is necessary to allow damaged cells to progress through to the next interphase (escaping cell cycle block). Following extended treatment without recovery (24 + 0 h -S9), all three cell lines demonstrated the same negative response to cigarette smoke. These data were not that surprising in the context of previous work conducted by Sobol et al. (2012) who observed that when using human lymphoblastoid TK6 cells, the extension of the recovery period from 2 to 3 cell cycles following short 4 h treatment, increased the magnitude of micronucleus induction for a number of chemicals. More recent work conducted by Whitwell et al. (2019) using human peripheral blood lymphocytes looked at a variety of clastogenic and aneugenic chemicals comparing extended (1.5–2 cell cycles) treatment with and without a recovery period (1.5–2 cell cycles). The results of these investigations showed that for the majority of chemicals tested the magnitude of MN induction was generally greater with MN induction observed across a wider concentration range following extended treatment with recovery. For some nucleoside analogues and aneugenic chemicals no MN response was detected using the extended treatment without recovery.

Therefore, the optimised conditions taken forward into Phase 2 for NGP assessment were as follows: V79 cells with the inclusion of the extended treatment with recovery period (24 + 24 h -S9), together with the standard 3 h ± S9 treatment conditions. The 24 + 0 h (-S9) treatment was omitted (being considered superfluous) for Phase 2. In Phase 2, NGP test articles were assessed in a comparable manner to cigarette smoke. Based on historical information, combustible cigarette smoke TPM was assessed using a 20 mg/mL TPM stock solution in DMSO, resulting in a top dose of 200 μg/mL (1% DMSO v/v for exposures) comparable to previously conducted studies (Combes et al., 2013; Crooks et al., 2013; Thorne et al., 2018a). THP and e-cigarette TPMs were generated up to 50 mg/mL, to achieve a final top dose concentration of 500 μg/mL (1% DMSO v/v in exposures). To further assess the potential of e-cigarettes to induce MNBN cells, the aerosolised matter was assessed in parallel to the native e-liquid at comparable doses up to 500 μg/mL. This assessment process allowed the e-liquid to be compared in its native form, and then upon aerosolization, thus capturing the transformation of this liquid and any associated chemicals potentially generated through the aerosolization process.

3R4F cigarette smoke TPM showed a positive induction in MNBN frequencies in all three treatment conditions. Positive responses were observed at 3 h -S9 in doses exceeding 180 μg/mL TPM. At 3 h (+S9), responses were observed at doses above 160 μg/mL TPM, and at 24 + 24 h -S9, with doses exceeding 120 μg/mL TPM. Some heterogeneity of response between replicate cultures was noted particularly at concentrations inducing high frequencies of MNBN cells following 24 + 24 h -S9 treatment, though this is not uncommon where positive responses are observed. Even where heterogeneity was apparent, this did not impact on the overall interpretation of the data as the general trends were clear and evaluation criteria were fulfilled.

In contrast, neither the THP (TPM) nor e-cigarette (TPM or e-liquid) produced a consistent or reproducible positive response even at the top dose of 500 μg/mL, as stated by ICH S2 (R1) (International Conference on Harmonisation, 2011). In some instances, the NGP test articles did

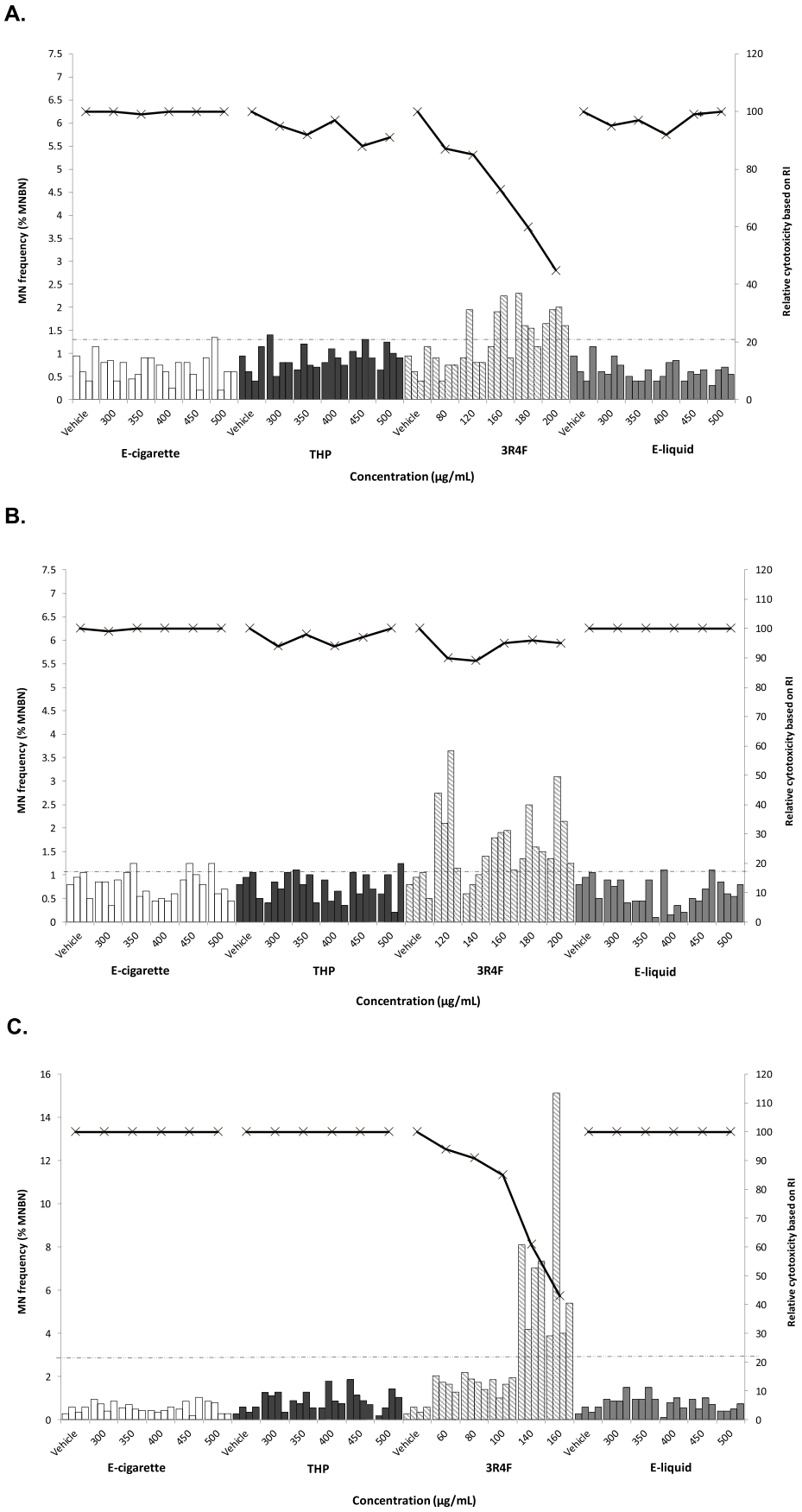

Fig. 5. V79 cell responses to 3R4F cigarette smoke and NGP test articles (THP, e-cigarette (TPM and e-liquid) under three treatment conditions, A) 3 + 21 h (-S9), B) 3 + 21 h (+S9) and C) 24 + 24 h (-S9). Though significant responses were observed for 3R4F smoke TPM under each condition, no induction of MNBN cells were observed for any NGP test articles. Bar charts show MN frequency of individual replicate cultures. Line graphs show relative cytotoxicity calculated based on RI. MN: micronucleus; MNBN: micronucleated binucleate cells; RI: replication index; dashed line = upper level of historical vehicle MNBN control range.

Table 5
Summary of responses to NGPs in V79 cells.

Condition	3 + 21 h (-S9)			3 + 21 h (+S9)			24 + 24 h (-S9)		
	Response	TPM or e-liquid (μg/mL)[a]	Nicotine (μg/mL)[b]	Response	TPM or e-liquid (μg/mL)[a]	Nicotine (μg/mL)[b]	Response	TPM or e-liquid (μg/mL)[a]	Nicotine (μg/mL)[b]
3R4F	Positive	160	7.96	Positive	140	6.97	Positive	140	7.96
THP	Negative	500	17.79	Negative	500	17.79	Negative	500	17.79
E-cigarette (TPM)	Negative	500	7.08	Negative	500	7.08	Negative	500	7.08
E-cigarette (e-liquid)	Negative	500	9.00	Negative	500	9.00	Negative	500	9.00

[a] = dose at first positive response or top dose with positive response.
[b] = corresponding nicotine dose.

not meet the required cytotoxicity threshold of 50–60% as described by OECD TG487 (OECD, 2016). Future studies should investigate increased dosing techniques. In terms of TPM production, this would require generating a higher stock concentration by capturing more particulate material onto the filter pad, or to investigate alternative TPM generation and extraction techniques. TPM has been generated in a consistent manner for many years, so any modifications to the standard methodology should be carefully considered. When using modified TPM preparation techniques, one should be mindful that any technique should at least match the characteristics of the standard TPM methodology. For example, stability of the TPM should be considered; that the capture or extraction process is equally as efficient as the standard methodology; and that any increases in particulate trapping does not adversely affect the ratios of chemicals present. Increased dosing should be contextualised against the OECD guideline for top-dose (recommended cytotoxicity, solubility/precipitation and solvent concentration) (OECD, 2016). In terms of e-liquids, in this study DMSO was used to normalise to traditional TPM extraction methods, but testing e-liquids neat or diluted in culture media is a very real and practical solution to increasing concentration, albeit within an accurate cellular osmotic range (Gonzalez-Suarez et al., 2017).

Although the top dose as defined by cytotoxicity may not have been reached for the NGPs, this study has demonstrated that the NGPs were tested at nicotine dose ranges equivalent to or greater than that of the cigarette TPM exposures, where positive responses were observed. This ensures that the NGPs were assessed within comparable ranges to cigarette smoke TPM when using nicotine as a marker of exposure.

Takahashi et al. (2018) tested THP TPM and demonstrated little to no activity in cytotoxicity (up to 1000 μg/mL using the neutral red uptake 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. Crooks et al. (2018) demonstrated increased dosing of a THP TPM compared to cigarette smoke and observed positive responses of THP TPM at approximately 1500 μg/mL in the IVMN assay using V79 cells. However, to achieve this concentration, cell cultures were dosed with up to 3% DMSO (v/v), higher than recommended in OECD guidelines (OECD, 2016), although no adverse effects were observed from this level of DMSO. These studies demonstrate that it is possible to achieve much higher TPM concentrations and generate positive responses from a THP.

This study assessed the response of multiple cell types to 3R4F cigarette smoke TPM and deemed V79 cells more responsive than CHO and TK6 cells for the assessment of cigarette smoke and NGP test matrices. Further investigation may be required into the choice of cell type and selection for the IVMN assay. In many respects, it will depend on regulatory requirements, the test article, the laboratory and their method validation procedures. However, this study does raise a valid question around the appropriate choice of cells for the IVMN, and how this assay can be applied when the response to the same cigarette smoke test article gave contrasting results. In this study, we selected the most sensitive cell line for the assessment of NGPs, by deeming the most responsive cell line to 3R4F TPM as being the most sensitive. Clearly, for comparative assessment purposes, cigarette smoke and NGPs should be assessed under the same conditions, including choice of cell type. In a flow cytometry IVMN study, Gao et al. (2016), showed little difference between CHO or BEAS-2B cells when assessing cigarette smoke TPM. The study suggested that multiple cell types were compatible with the IVMN assay for TPM and e-liquid assessments. At this stage, it is unclear why the cell lines assessed in our study demonstrated varying responsiveness to the 3R4F TPM. Potentially, it may be as simple as protocol optimisation as suggested by Gao et al. (2016).

Possible differences in response between the human TK6 and the rodent V79, CHO cell lines may be linked to p53 status. Earlier investigations by Fowler et al. (2012) looked at the response to 19 ′misleading positive' chemicals (positive in mammalian cell assays but which have been shown to be non-DNA reactive, Ames and *in vivo* negative (Kirkland et al. (2008)), in several commonly used p53-deficient hamster cell lines (V79, CHO, CHL) as well as p53-competent human cells (primary human lymphocytes, TK6 lymphoblastoid cells, and HepG2 cells). Results suggested that rodent p53 deficient cells were more prone to providing a misleading positive response than human (p53 competent) cell lines and it was considered that p53 compromised cell lines may be deficient in necessary checkpoint control over DNA damage, allowing cells to progress through mitosis and exhibit micronuclei rather than undergoing cell cycle arrest, apoptosis (or necrosis) and cell death. However, further follow-up work conducted by Whitwell et al. (2015) looking at the relationships between p53 status, apoptosis and induction of micronuclei in different human (p53 competent and p53 compromised) and mouse (p53 compromised) cell lines found that p53 status was not the primary factor and that apoptosis and potential differences in DNA repair capabilities between rodent and human cell lines had a role to play. In the context of these investigations, although the various p53 and rodent, non-rodent status of the cell types used were considered, (all being regulatory OECD 487 acceptable), it was their ability and usefulness to discern between cigarette smoke and NGP test matrices that was important.

Future studies could also consider assessing the whole aerosol, using IVMN whole aerosol methodologies. However, these methodologies are not routinely used, suggesting these methods are still in their infancy compared to other whole aerosol methodologies, and more method development is required to implement them as part of a whole aerosol testing strategy. Most IVMN studies on cigarette smoke (and on limited occasions with NGPs) have been conducted on TPM or the captured gas vapour phase in aqueous solution (Crooks et al., 2015, 2018; Gao et al., 2016; Misra et al., 2014; Roemer et al., 2015; Scott et al., 2013; Takahashi et al., 2018), further supporting this observation that WA methodologies are not as mature as TPM techniques.

Finally, this study has demonstrated that by using an extended treatment with a recovery period of 1.5–2 cell cycles (24 h for V79 and CHO cells), the dynamic range of the assay has been increased. This was evident in both V79 and CHO cells, which demonstrated increased responses to cigarette smoke with the application of an additional 24 h recovery period. No increase in dynamic range was observed in TK6

cells, which showed predominately weak-positive responses. This observation may be of particular importance when assessing NGPs, where the 24 h recovery period may give additional dynamic range. In this study, the NGPs were deemed negative in the IVMN assay. Future NGP studies should consider the application of an increased recovery period, to ensure optimal conditions for NGP assessment and potentially combined with increased TPM doses where feasible. This combination may even give increased differentiating potential of the assay when assessing NGPs. However, in the absence of a positive response, this remains only a hypothesis and requires further investigation.

5. Conclusions

This study has demonstrated a clear and reproducible cigarette smoke dose response for the induction of micronucleus frequencies in three treatment conditions using V79 cells. V79 cells were determined to be more sensitive to cigarette smoke exposure in this study, when compared to CHO and TK6 cells. At TPM doses far exceeding those of cigarette smoke, and at equivalent or greater nicotine dose levels, THP and e-cigarette test matrices were deemed negative in inducing micronuclei. With respect to e-cigarettes, this observation is reassuring, giving the relatively simple makeup of e-liquids (propylene glycol, Vegetable glycerol, water, nicotine and some flavourings depending on formulation).

Finally, this is Part Two of a two-part series where the same test articles and TPM preparations were compared in both the MLA and the IVMN assay (IVMN detailed here), in a co-ordinated genotoxicity testing approach. Part one of this series (Thorne et al., 2018b) also compared the same test articles (same TPM and e-liquid preparations) in the MLA assay and demonstrated negative results in response to NGPs and a consistent response to 3R4F cigarette smoke TPM. In contrast, cigarette smoke produced a positive response under all treatment conditions. Despite the surrounding questions on cell type, the data between these two studies are reassuring in that both the MLA and IVMN assays, when evaluated with the same test articles, produced consistent negative responses to NGP TPMs and positive response for 3R4F cigarette smoke, further supporting the use of either the MLA or IVMN assay as part of a recommended test battery.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors contributions

The study was designed by, Marianna Gaça, Betsy Bombick, David Thorne, Damien Breheny and Robert Leverette. The experimental work was conducted, analysed and overseen by, subject matter experts at Covance Laboratories, Mel Lloyd, James Whitwell and Steven McEnaney. Marianna Gaça, Betsy Bombick and Julie Clements oversaw all biological testing for British American Tobacco, RAI Services Company and Covance respectively. All authors drafted and approved the final version.

Conflicts of interest

The work was jointly funded by British American Tobacco (BAT) and RAI Services Company (RAIS). RAIS is a member of the BAT group. Covance laboratories conducted all the work at their Harrogate, Genetic Toxicology Facility in the UK and was funded by BAT and RAIS. All authors were employees of either BAT, RAIS or Covance Laboratories at the time of study conduct.

Acknowledgements

Kathy Fowler for her oversight and technical contributions to this study. Kristen Jordan for her technical and editorial review and Amy Wakefield for her logistics planning and support.

Abbreviations

DMSO	dimethyl sulphoxide
E-cigarette	electronic cigarette
E-liquid	electronic cigarette liquid
HCI	Health Canada Intense smoking regimen
IVMN	*in vitro* micronucleus assay
MNBN	micronucleated binucleate
NGP	next generation products
THP	tobacco heating product
TPM	total particulate matter
S9	mammalian liver post-mitochondrial fraction

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.05.054.

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