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Assessment of novel tobacco heating product THP1.0. Part 7: Comparative *in vitro* toxicological evaluation

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ABSTRACT

In vitro studies have been widely used to support the toxicological evaluation of chemicals and complex mixtures including cigarette smoke. In this study, the total particulate matter and whole aerosol from a Kentucky reference 3R4F cigarette and two commercially available tobacco heating products (THPs) were assessed using *in vitro* mutagenicity, cytotoxicity and tumour-promoting activity assays. The Ames assay assessed mutagenicity using *Salmonella typhimurium* tester strains TA98, TA100, TA1535, TA1537 and TA102 ± metabolic activation (S9). The mouse lymphoma assay was used with short 3 h and longer 24 h exposures. The Bhas 42 cell transformation assay was incorporated as an *in vitro* alternative for detecting tumour promoters, and the neutral red uptake cell viability assay provided an acute measure of cytotoxicity. To complement the approach, the Ames assay was also employed with *S. typhimurium* tester strains TA98, TA100, TA1535, TA97 and TA102 using a scaled down methodology for the assessment of aerosols. All the *in vitro* techniques employed produced a clear positive response with cigarette smoke and in contrast, a negative response to THPs at doses equivalent to or higher than a cigarette smoke test matrix. The data show little difference between the THPs assessed suggesting parity between products.

1. Introduction

Cigarette smoking is a major risk factor for many adverse health conditions, including cardiovascular disease, respiratory disease and lung cancer (Stratton et al., 2001; US DHHS, 2014). Given these effects, the tobacco industry has spent many years investigating reduced exposure technologies, cigarettes and devices to limit toxicant exposure in those that continue to smoke Institute of Medicine (2011). The chemical composition of smoke from any product or device results from the choice of tobacco blend, the design and/or format and the presence or absence of a filter and filter components, such as charcoal and/or other selective adsorptive materials. Recent examples of technologies aimed at reducing toxicant profiles include substitute tobacco sheet, which acts as a tobacco diluent (McAdam et al., 2001); the development and refinement of cigarette design, format and selective filtration (Bombick et al., 1997; Branton et al., 2011; Dittrich et al., 2014); treatment of tobacco prior to cigarette manufacturing (Liu et al., 2011); agronomic practices (Lewis et al., 2008); and the development of alternative products, such as electronic cigarettes (e-cigarettes) and heat not burn devices (Doolittle et al., 1990; Foy et al., 2004; Goniewicz et al., 2014; R. J. Reynolds Tobacco Company, 1988; Schaller et al., 2016; Smith et al., 1996, 2016).

Next-generation tobacco and nicotine products (NGPs), such as tobacco heating products (THPs) and e-cigarettes, have evolved significantly over recent years and are reaching consumer acceptability. THPs operate by heating a tobacco rod to temperatures up to approximately 350 °C, which is significantly lower than that found during the combustion of cigarettes (>900 °C). At these lower temperatures, the aerosol generated has a less-complex chemical composition compared to that of a conventional cigarette (Eaton et al., 2017; Forster et al., 2017; Schaller et al., 2016). Recent studies have demonstrated that THPs had significantly reduced levels of harmful constituents when compared to a 3R4F reference cigarette (Forster et al., 2017; R. J. Reynolds Tobacco Company 1988; Smith et al., 2016) and have demonstrated reduced toxicity in laboratory-based *in vitro* tests (Breheny et al., 2014; Schaller et al., 2016). Given the development of NGPs, there is a requirement to assess these emerging products to understand how they compare to conventional tobacco products.

As part of an assessment strategy investigating the reduced exposure potential of NGPs, non-clinical testing, including chemical analysis and *in vitro* toxicity testing, can be employed initially as

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Abbreviations

Bhas	Bhas 42 cell transformation assay
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulphoxide
GEF	global evaluation factor
HCI	Health Canada Intense
HCIm	Health Canada Intense modified
MEM	Modified Eagles medium
MLA	Mouse Lymphoma Assay
OECD	Organization for Economic Cooperation and Development
S9	rat liver metabolic activation system
TPA	12-O-tetradecanoylphorbol-13-acetate
TPM	total particulate matter
THP	Tobacco Heating Product
THP1.0	Tobacco Heating Product version 1
THS	Tobacco Heating System
3R4F	University of Kentucky reference cigarette

part of a stewardship approach (Liu et al., 2011; Murphy et al., 2017). As a complement to chemical analysis of emissions (Eaton et al., 2017; Forster et al., 2017, Schaller et al., 2016), a battery of *in vitro* toxicity tests may be used for initial screening of the mutagenic and cytotoxic potential of NGPs (Murphy et al., 2017; Schaller et al., 2016). International guidelines have been developed that recommend an appropriate battery of *in vitro* mutagenicity and carcinogenicity assays to ensure consistency of testing procedures and appropriate assay selection as part of a risk assessment process. Several guidelines exist, including those developed by the International Conference on Harmonisation (ICH 2011), the Committee on Mutagenicity (COM 2011), Health Canada (Health Canada, 2005) and the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA, 2004). In summary, these guidelines recommend the use of i) a bacterial mutagenicity assay (Ames reverse mutation assay), (Maron and Ames, 1983); ii) a mammalian cell based assay for cytogenetics/mutation (*in vitro* micronucleus assay) (Parry et al., 2002); iii) chromosome aberrations or the mouse lymphoma assay (MLA), (Hozier et al., 1981)]; and iv) a cytotoxicity-based assay. The Bhas 42 cell transformation assay is often used to supplement testing approaches and add to a weight of evidence approach for *in vitro* carcinogenicity testing. Although the Bhas 42 cell transformation assay is not a recognised assay for use in regulatory testing, a guidance document has been issued by the Organization for Economic Cooperation and Development (OECD) (OECD, 2016). This assay is considered to add value in its ability to detect non-genotoxic carcinogens and to support a weight of evidence based testing strategy.

The NRU, Ames and MLA assays are used routinely to assess total particulate matter (TPM) from cigarette smoke, and have been extensively employed (Andreoli et al., 2003). The responses from cigarette smoke TPM in these assays have been ubiquitously positive using reference cigarette smoke test articles (Combes et al., 2013; Crooks et al., 2013; Scott et al., 2013). Additionally, these assays have been used in an assessment strategy to compare traditional combustible cigarettes and have shown distinguishing potential (Combes et al., 2012). Furthermore, these techniques and approaches have been used to assess the genotoxic and cytotoxic potential of NGPs including e-cigarettes and THPs (Azzopardi et al., 2016; Foy et al., 2004; Schaller et al., 2016; Thorne et al., 2016). The results from these assays have shown that these products are either negative or extremely low-responding compared to traditional tobacco smoke, which appears to correlate with the reduction in levels of chemical compounds and toxicants found in the source aerosol compared to tobacco smoke.

In this study, TPM from a Kentucky reference cigarette (3R4F) was compared to TPM generated from two THPs, commercially available in Japan: Tobacco Heating Product version 1 (THP1.0) and Tobacco Heating System (THS). The NRU assay was employed for assessment of acute cytotoxicity; the Ames assay as a measure of bacterial mutagenicity, the MLA as a mammalian mutagenicity test and supplemented with the Bhas 42 cell transformation assay. To further complement these studies, whole aerosols were assessed from the three products using the Ames assay and a scaled-down 35 mm agar plate methodology.

2. Materials and methods

2.1. Chemicals and reagents

Where specified, Aroclor-1254-induced rat liver post-mitochondrial supernatant (S9) mix (Moltox™, Boone, NC, USA) provided metabolic activation. All other chemicals were obtained from Sigma-Aldrich (Dorset, UK), unless otherwise stated.

2.2. Products

Three products were assessed in this study; a scientific reference cigarette (3R4F) and two commercially available tobacco heating products: THP1.0 and THS sourced from Japan. An overview of specifications for the three products assessed in this study are provided in Table 1, and more technical product details on consumables and emission chemistry data are detailed for the THP1.0 in Eaton et al. (2017) and for THS in Schaller et al. (2016).

2.3. Total particulate matter (TPM) generation

TPM was generated in a comparable manner for each product. Reference 3R4F cigarettes and THP consumables were conditioned as per International Organization for Standardization (ISO) guideline 3402:1999 (ISO, 1999) and puffed, respectively, on a Borgwaldt RM200A2 and a Borgwaldt LM20X linear machine (Borgwaldt-KC, Hamburg, Germany). The Health Canada Intense (HCI) smoking regime (55 ml puff volume, 2 s puff duration and 30 s puff interval, 100% vent blocking; Health Canada Official Method T-115 (HCI 1999)) was used for 3R4F, and a modified HCI regime modified (HCI_m) without vent blocking, which is not possible for THPs. Up to 150 mg TPM was collected onto 44 mm Cambridge filter pads (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 concentration of 24 mg/ml. TPMs were stored in single use aliquots at −80 °C.

For all products, 'partner' pads were smoked/puffed on each day of TPM generation. In addition, for every 10 3R4F pads collected, a quality control (QC) pad was also collected under the ISO condition. Partner and QC pads were sent to the BioPharmaceutical CMC Solutions – small molecules Department (Covance, Harrogate, UK) for analysis of nicotine, water and glycerol content by GC-TCG and GC-FID.

A summary of the test article TPM characterisation can be found in Table 2.

2.4. Whole aerosol (WA) exposure

Two Vitrocell VC 10 smoking robots (Vitrocell systems, Waldkirch, Germany), serial numbers VC10/090610 and VC10/060614,

Table 1
Overview of product specifications.

Parameter	Product		
	3R4F reference cigarette[a]	THP1.0[b]	THS[c]
Product	Scientific 3R4F research reference cigarette	Commercial THP	Commercial THP
Aerosol-generation principle	Combustion, distillation and condensation	Distillation and condensation	Distillation and condensation
Aerosolisation temperature (°C)	<900[d]	<250	<350
Smoking time (min)	5–8	4	6
Number of puffs	5–10	8	12
Smoking regimen	Health Canada Intense[e]	Modified Health Canada Intense (no vent blocking)	Modified Health Canada Intense (no vent blocking)
Smoking profile	Bell	Bell	Bell

[a] Roemer et al., 2012.
[b] Eaton et al., 2017.
[c] Smith et al., 2016.
[d] Baker, 2006.
[e] Health Canada Official Method T-115 (puff volume 55 ml, puff interval 30 s, puff duration 2 s, 100% vent blocking).

Table 2
Total particulate matter characterisation.[a]

Product	Glycerol content (mg/cig)	Nicotine content (mg/cig)	Water content (mg/cig)	NFDPM(mg/cig)
3R4F	2.3 ± 0.1	1.9 ± 0.1	12.0 ± 0.5	24.3 ± 0.8
THP1.0	2.7 ± 0.2	0.4 ± 0.02	8.5 ± 0.6	6.0 ± 0.7
THS	3.6 ± 0.3	1.1 ± 0.04	15.9 ± 1.0	10.0 ± 0.6

Data are mean (±standard deviation).
Abbreviation: NFDPM = nicotine-free dry particulate matter.
[a] International Organization for Standardization guideline 3402:1999 [ISO 1999].

were used to expose bacteria to freshly generated aerosols. To eliminate cross-aerosol contamination, VC10/090610 was used for 3R4F exposure and VC10/060614 was used for THP (THP1.0 and THS) exposures across the study. In an inter-laboratory study, Adamson et al. (2014) demonstrated good consistency between six independent VC 10 machines (Gauge R&r 7.7%). Therefore, using two systems in a study such as this was not considered an issue, and was preferable to cross-aerosol contamination. Aerosol dilution in the VC 10 is achieved via mixing of the aerosol in the dilution bar via the additional of a perpendicular air flow, which creates a turbulent homogenous aerosol. Different aerosol concentrations are achieved by increasing or decreasing the diluting airflow. A vacuum is used to sample the aerosol (via negative pressure) from the dilution bar into the module, which docks directly under the dilution bar (Fig. 1). Diluting airflow rates within this system were maintained using mass flow controllers (Analyt-MTC GmbH, Mülheim, Germany).

Triplicate bacterial plates were exposed in Vitrocell AMES 4 stainless steel exposure modules. The trumpet height within the module was set to 2 mm above the agar surface. Diluting airflows of 12, 8, 4 and 1 l/min with a fixed vacuum of 5.0 ml/min/well, were assessed. Ames plates were exposed to diluted 3R4F cigarette smoke and THP aerosols between 24 and 180 min. Exposure times were determined based on previous studies (Thorne et al., 2015; Thorne et al., 2016).

The Vitrocell VC 10 smoking robot has been extensively characterised, and is detailed in numerous studies for the assessment of a variety of aerosols, including cigarette smoke, individual gases and aerosols from THPs and e-cigarettes, (Breheny et al., 2014; Ishikawa et al., 2016; Majeed et al., 2014; Thorne et al., 2013, 2016).

2.5. Measurement of deposited particulate mass during aerosol exposure

To define exposure conditions, one quartz crystal microbalance (QCM) (Vitrocell Systems) was installed into the last position of the Ames 4 stainless steel exposure module. QCM technology has been demonstrated to be a viable tool for the assessment of dose (μg/cm^2) *in situ* (Adamson et al., 2014; Adamson et al., 2016; Majeed et al., 2014).

2.6. Biological techniques

A summary of all the biological tests employed can be found in Table 3 along with their protocol ID or OECD test guideline/guidance document references. Although an independent cytotoxicity assessment was carried out in the form of NRU, each assay, Ames, MLA and Bhas, contained its own cytotoxicity assessment assessed alongside each endpoint. All *in vitro* assessments were conducted in accordance with Good Laboratory Practice. All data can be found in supplementary materials.

2.7. Neutral red uptake (NRU) assay

Mouse fibroblast cells (Balb/c 3T3 Clone A31) were obtained from the European Collection of cell cultures. All testing was based on published guidance from The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) NIH Publication No. 07-4519 (ICCVAM, 2006). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) complete, supplemented with 10% foetal bovine serum (FBS), 4 mM L-glutamine and penicillin or streptomycin at 37 °C ± 1 °C in a humidified atmosphere (5% CO_2). Cells were routinely screened for mycoplasma contamination.

Prior to exposure, cells were seeded into 96-well culture plates at 1×10^5 cells/ml in 100 μl and maintained for 48 h to establish a semi-confluent monolayer. Cells were then washed with phosphate buffered saline (PBS), and exposed for 24 h to eight concentrations, and six replicates per concentration of test material suspended in DMEM complete, over four or more independent experiments.

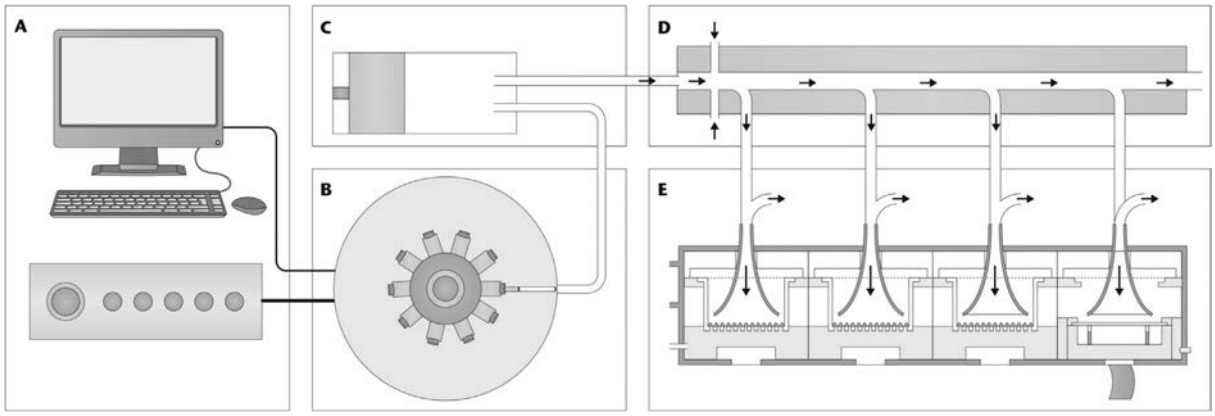

Fig. 1. A schematic representation of the Vitrocell VC 10 smoking robot. (a) Software and air-flow controller, (b) Smoking and ventilation hood for aerosol generation. (c) Piston, which draws the puff and delivers the aerosol to the dilution system. (d) Dilution, transit and delivery of aerosol occurs in the dilution system (bar). (e) Exposure module, which holds the scaled-down 35 mm Ames plates with quartz crystal microbalance installed into position 4 within the module. Figure adapted from Thorne et al. (2013).

Table 3
Summary of assays used for *in vitro* biological assessment.

Endpoint	Technique	Cell/Bacterial System	Metabolic activation	Guideline/protocol
Cytotoxicity	Neutral red uptake assay	BALB/c 3T3 mouse fibroblasts	None	ICCVAM (NIH Publication No. 07-4519)
Mutation	Bacterial reverse mutation (Ames) assay[a]	*Salmonella typhimurium* TA98 TA100 TA1535 TA1537 TA102	±S9 (preincubation and plate incorporation)	OECD 471
	Bacterial reverse mutation (Ames) assay[b]	*Salmonella typhimurium* TA98 TA100 TA1535 TA97 TA102	+S9 (scaled-down 35 mm spread plate methodology)	OECD 471[c]
	Mouse lymphoma assay *(in vitro* gene mutation assay at the $tk^{+/-}$ locus)	Mouse lymphoma L5178Y cells	±S9	OECD 490
Cell transformation (tumour promotion)	Bhas 42 cell transformation assay (promotion protocol)	Bhas 42 mouse embryo fibroblast cells	None	OECD guideline issued[d]

Abbreviations: ICCVAM= Interagency Coordinating Committee on the Validation of Alternative Methods; NIH=US National Institutes of Health.
[a] Uses total particulate matter.
[b] Uses whole aerosol.
[c] Adapted method.
[d] currently under discussion.

Following the 24 h exposure period, the cells were washed with PBS and 100 μl of neutral red (NR) solution was applied to the plate (50 μg/ml) and incubated for 3 h. The NR absorbance was read at optical density 540 nm (OD_{540}). For each concentration, the resulting reduction in 50% toxicity (IC_{50}) were calculated. Where an IC_{50} could not be calculated, the test article was classified as 'non-cytotoxic'. The following acceptance criteria were applied to all assessments: for control treatments, the observed variability (the coefficient of variance) in OD_{540} values should be <15%; the positive control treatment (sodium dodecyl sulphate 100 μg/ml) should cause >50% decrease in cell death, relative to the control; the mean OD of the vehicle control should be > 0.2 (OD_{540}). Toxicity profiles were calculated using Graphpad Prism (7) with variable 4 parameter slope analysis.

2.8. Bacterial reverse-mutation assay culture conditions

Prior to optimisation of culture conditions, each strain was checked for strain characteristics and antibiotic resistance (De Serres and Shelby, 1979; Maron and Ames, 1983). In brief, bacteria were cultured at 37 ± 1 °C in an anhydric incubator in nutrient broth containing appropriate nutrients to obtain bacterial culture with densities of 10^8–10^9 cell/ml, for approximately 10 h. Treatments were conducted within 6 h of incubation.

2.9. Bacterial reverse mutation assay with TPM

To evaluate TPM, five tester strains of *S.typhimurium* (TA98, TA100, TA1535, TA1537, TA102) were used in the presence and absence of S9 in accordance with OECD guideline 471 [OECD, 1997]. Appropriate positive and vehicle controls were included in all assays. At least eight concentrations of TPM per product were used per assay. Independent repeat plate-incorporation and pre-incubation tests were performed on each strain.

2.10. Bacterial reverse mutation assay with WA

The Ames methodology used in this study is modified from the standard 85 mm plate methodology and has been previously described (Kilford et al., 2014; Thorne et al., 2015, 2016). WA

exposures were performed in a scaled-down 35 mm plate format (Grenier Bio-One). Approximately 2×10^7 bacteria cells were mixed with 75 μl sodium phosphate buffer (pH 7.4) or a 10% S9 mix, and complemented with 40 μg/ml histidine and 48.8 μg/ml biotin mix. The bacterial cell suspension was plated directly on to Vogel-Bonner agar using a spread plate technique and incubated at 37 °C until dry before transferring to Vitrocell AMES 4 exposure module.

Concurrent controls (air, untreated and positive) were included with each exposure. Air controls were exposed to a constant flow of filtered air (0.2 l/min diluting air flow, 5.0 ml/min/well vacuum). Following exposure, the plates were removed from the exposure modules, sealed, inverted and incubated at 37 °C in the dark for 3 days. Cigarette smoke exposures were conducted for up to 24 min, whereas the THP aerosol exposures were conducted for up to 3 h. To evaluate aerosol, five tester strains of *S. typhimurium,* (TA98, TA100, TA1535, TA97 and TA102) were used in the presence and absence of S9, in accordance with OECD guideline 471.

For an increase in revertant numbers to be considered as a mutagenic response in the Ames assays, increases were required to be statistically significant ($p = 0.01$) using Dunnett's test, and both concentration-related and reproducible over two or more independent experiments as per OECD 471 guidelines.

A complete breakdown of the strains and positive controls used in both the TPM and whole aerosol assays is shown in Table 4.

2.11. Mouse lymphoma assay

Mouse lymphoma cells (L5178Y $tk^{+/-}$) were originally sourced from Burroughs Wellcome & Co, Dartford, UK. All testing was conducted in accordance with OECD guideline 490 (OECD, 2015). TPM from all three products were assessed under three test conditions, 3 h with or without S9 and 24 h without S9. Cell cultures were maintained in Roswell Park Memorial Institute medium (RPMI)-10 cell culture media (RPMI-1640, supplemented with heat-inactivated horse serum [10%] and penicillin or streptomycin) at 37 °C ± 1 °C. For exposure, at least 1×10^7 cells and/or 4×10^6 cells for the 3 h and 24 h exposure., were placed in centrifuge tubes (3 h) or culture flasks (24 h) and test article, positive control or negative control were added. Following exposure periods cells were centrifuged (200 *g*) washed and transferred to tissue culture flasks for the expression period. Cultures were maintained for 48 h to assess L5178Y $tk^{+/-}$ expression. At the end of the expression period, cells were plated for either viability assessments or mutation assessments. For each assessment, plates were incubated for up to 2 weeks at 37 °C ± 1 °C in a humidified atmosphere (5% CO_2). The number of wells containing large and small colonies were scored for all treatments, and where the sum of any treatments exceeded the mutant frequencies (MF) of the vehicle control and global evaluation factor (GEF). The data were classified as mutagenic if the following conditions were met: the MF of any test concentration exceeded the sum of the vehicle control MF plus the GEF or the linear trend was statistically significant (determined by linear fit analysis).

2.12. Bhas cell transformation assay

Bhas 42 mouse fibroblast cells (obtained from Hatano Research Institute, Food and Drug Safety Centre, Japan Cell Bank, Ochiai, Japan, were cultured in modified Eagle's medium (MEM), (Gibco) supplemented with 5% FBS and 50,000 U penicillin and 50 mg streptomycin. Three days before treatment, cells were transferred to DMEM/F12 (Gibco) supplemented with 5% FBS. Prior to the initiation of promotion assay, a single cell-growth assay was performed to identify test concentrations for the promotion assay (those concentrations resulting in viability of 50% and above). The

Table 4
Strain characteristics, sources and positive controls.

Salmonella typhimurium strain	Antibiotic resistance	Plasmid	Mutation	Mutant gene	Test matrix[a]	Positive control treatments			
						S9	Compound	Final concentration 85 mm (μg/plate)	Final concentration 35 mm (μg/plate)
TA98[b]	Ampicillin	pKM101	Frame-shift	Histidine	TPM & WA	−	2-Nitrofluorene	5	0.4
						+	Benzo(a)pyrene	10	0.8
TA100[c]	Ampicillin	pKM101	Base-pair substitution	Histidine	TPM & WA	−	Sodium azide	2	1
						+	2-Aminoanthracene	5	0.4
TA1535[b]	None	pKM101	Base-pair substitution	Histidine	TPM & WA	−	Sodium azide	2	0.8
						+	2-Aminoanthracene	5	0.8
TA1537[b]	None	pKM101	Frame-shift	Histidine	TPM	−	9-Aminoacridine	50	N/A
						+	2-Aminoanthracene	5	N/A
TA97[d]	Ampicillin	pKM101	Base-pair substitution	Histidine	WA[e]	−	ICR-191	N/A	0.3
						+	2-Aminoanthracene	N/A	0.8
TA102[c]	Ampicillin & tetracycline	pKm101 pAQ1	Base-pair substitution	Tryptophan	TPM & WA	−	Mitomycin C	0.2	1.0
						+	2-Aminoanthracene	20	3.2

Abbreviations: TPM = total particulate matter; WA = whole aerosol.
[a] TPM used with 85 mm plate incorporation and pre-incubation techniques and WA used with scaled-down 35 mm spread-plate methodology.
[b] National Collection of Type Cultures, Porton Down, Salisbury, UK.
[c] Covance Laboratories Inc., USA.
[d] Prof. Ames' laboratory.
[e] TA97 was used instead of TA1537 in the scaled down spread plate format.

promotion assay was carried out as shown in Fig. 2 and described previously (Weisensee et al., 2013). In brief, 14,000 cells per well were seeded and maintained in six well plates for 3 days. A total of six wells per treatment were used. Cells were treated with test solutions for 10 days, with a change to fresh treatment medium on days 7 and 10. On day 14, treatment medium was replaced with fresh DMEM/F12 supplemented with 10% FBS and left to incubate for a further 7 days, after which the cells were fixed with methanol. Solvent controls (DMSO without TPM) and positive controls (12-O-tetradecanoylphorbol-13-acetate (TPA) in DMSO, 50 ng/ml) were treated in the same manner. A test was considered valid if the positive control response was statistically significant compared to the vehicle control ($p = 0.05$). A cell-growth assay was carried out in parallel to each promoter experiment (OECD guideline 251 (OECD, 2016)). A total of three wells per treatment group were used. A one-sided ANOVA with *post hoc* Dunnett's test was applied to the data (significance level set at $p = 0.05$). The treatment groups for each test product were compared against the response of the DMSO vehicle control.

2.13. Statistics and data analysis

All analyses were conducted in line with the experimental approach as discussed under each section. Data are presented for each endpoint as mean ± standard deviation (±SD).

3. Results

3.1. NRU assay

Fig. 3 shows the responses to TPM from all products in the NRU assay following exposure up to 240 μg/ml.

3R4F TPM showed a dose-responsive decrease in viability using the Balb/c 3T3 NRU cytotoxicity assay at doses between 20 and 140 μg/ml. Complete cellular death was observed at 140 μg/ml, with an IC_{50} calculated at 81.2 μg/ml. The dose response was consistent between all five independent experiments ($R^2 = 0.93$). Neither THPs (THP1.0 or THS) showed a decrease in viability at any of the TPM concentrations tested up to the maximum achievable dose of 240 μg/ml. Under the evaluation criteria, any test article failing to achieve at last a 50% decrease in viability were considered non-cytotoxic.

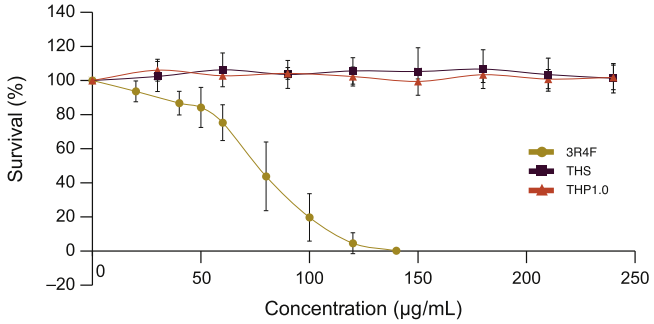

Fig. 3. Acute cytotoxicity in the NRU assay using BALB/c 3T3s. A positive cytotoxic response was observed with 3R4F cigarette smoke, and non-cytotoxic responses were observed with both THPs.

3.2. Ames bacterial reverse-mutation assay with TPM

Fig. 4 shows the responses in the Ames assay to TPM from all products in the presence of metabolic activation only (data in the absence of metabolic activation can be found in supplementary material).

Following 3R4F TPM treatments, concentration-related and reproducible increases in revertant numbers were observed that were statistically significant at the 1% level when analysed by Dunnett's test. Increases were evident in strains TA98, TA100 and TA1537 in the presence of S9, with smaller increases seen in strains TA98 and TA100 in the absence of S9. These increases indicate that 3R4F TPM has mutagenic activity in these strains in the presence of S9 and in strains TA98 and TA100 in the absence of S9. Statistically significant increases ($p = 0.01$ in Dunnett's test) were observed in other strains across treatment conditions and experiments. However, these increases were not reproducible over all treatment occasions, and were not concentration-related, and therefore were not considered as demonstrating mutagenic activity in the test article.

Generally, evidence of toxicity (thinning of the background bacterial lawn or a reduction in bacteria) in 3R4F TPM treatments were observed at the highest concentrations in all strains in the absence of S9, and to some extent in the presence of S9. The mutagenic response tailed off at the highest concentrations (TPM 2400 μg/plate) in strains TA98, TA100 and TA1537 in the presence of S9, which were further indications of toxicity. No clear evidence

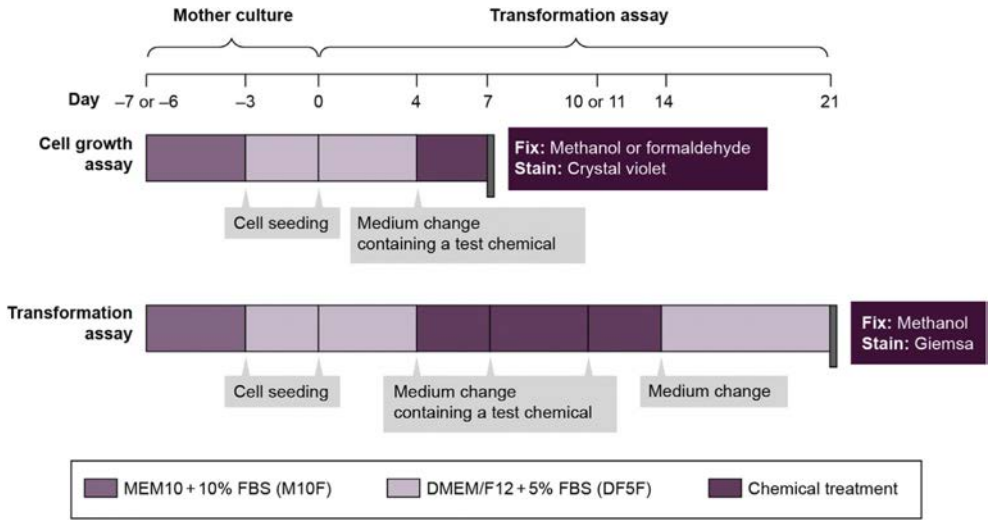

Fig. 2. Schematic representation of cell-growth assay and promoter-transformation assay protocol used in this study. [OECD guidance document 2016] Abbreviations: MEM = modified Eagle's medium. DMEM = Dulbecco's modified Eagle's medium.

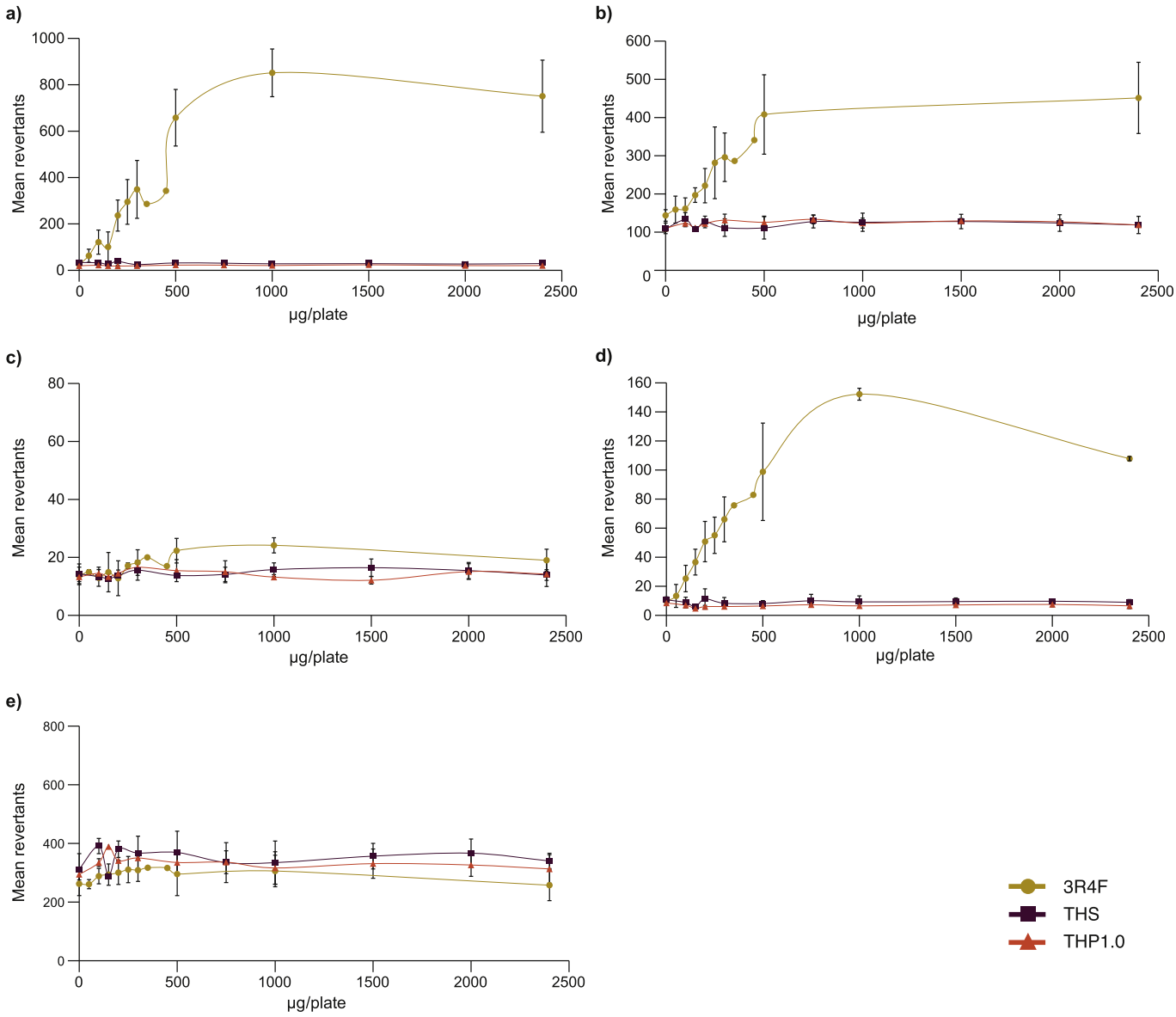

Fig. 4. Reverse mutations using Ames in strains (a) TA98, (b) TA100, (c) TA1535, (d) TA1537 and (e) TA102 in the presence of metabolic activation for all three products assessed in response to TPM. Mutagenic response can be observed for cigarette smoke in TA98, TA100 and TA1537 ($p = 0.01$). Both THPs were deemed non-mutagenic under all test conditions.

of toxicity or mutagenicity was observed for either of the two THP TPMs tested.

The data show in the presence of metabolic activation that 3R4F TPM is mutagenic in tester strains TA98, TA100 and TA1537. The data were reproducible, dose-responsive and significant using the Dunnett's test to $p = 0.01$. Conversely, cigarette smoke was deemed non-mutagenic in tester strains TA1535, TA1537 and TA102 in the absence of metabolic activation. Both the THP TPMs from THP1.0 and THS were deemed non-mutagenic in all strains in the presence and absence of metabolic activation. No significant reproducible or dose responsive increases were observed above the vehicle control in any test condition. No difference was observed between the two commercially available THPs and neither product differentiated from the untreated control.

3.3. Bacterial reverse-mutation assay with WA

Fig. 5 shows the responses in the Ames assay to WA from all products in the presence of metabolic activation.

S. typhimurium tester strains TA98, TA100, TA1535, TA97 and TA102 in the presence of metabolic activation were used to assess WA from 3R4F and both THPs, using a scaled-down 35 mm spread plate methodology previously described (Kilford et al., 2014; Thorne et al., 2015, 2016). 3R4F cigarette smoke was deemed positive in tester strains TA98 and TA100 in as little as 24 min at the highest concentration of aerosol tested, which equated to an airflow of 1 l/min. 3R4F cigarette smoke aerosol dilutions 12, 8, 4 and 1 l/min were used with concurrent air and untreated controls. Deposited mass (µg/cm^2) was used to assess dose *in situ* within the exposure module. Revertant data are presented as a function of dose to allow extrapolations to historic WA studies and not according to an arbitrary dilution principle (l/min). In tester strain TA98 and TA100, a positive response was observed at 2.9 and 3.3 µg/cm^2, corresponding to a 24 min exposure at 4 l/min. Thinning of the background lawn was observed in all strains at 1 l/min after a 24 min exposure. Conversely, neither THP1.0 or THS were

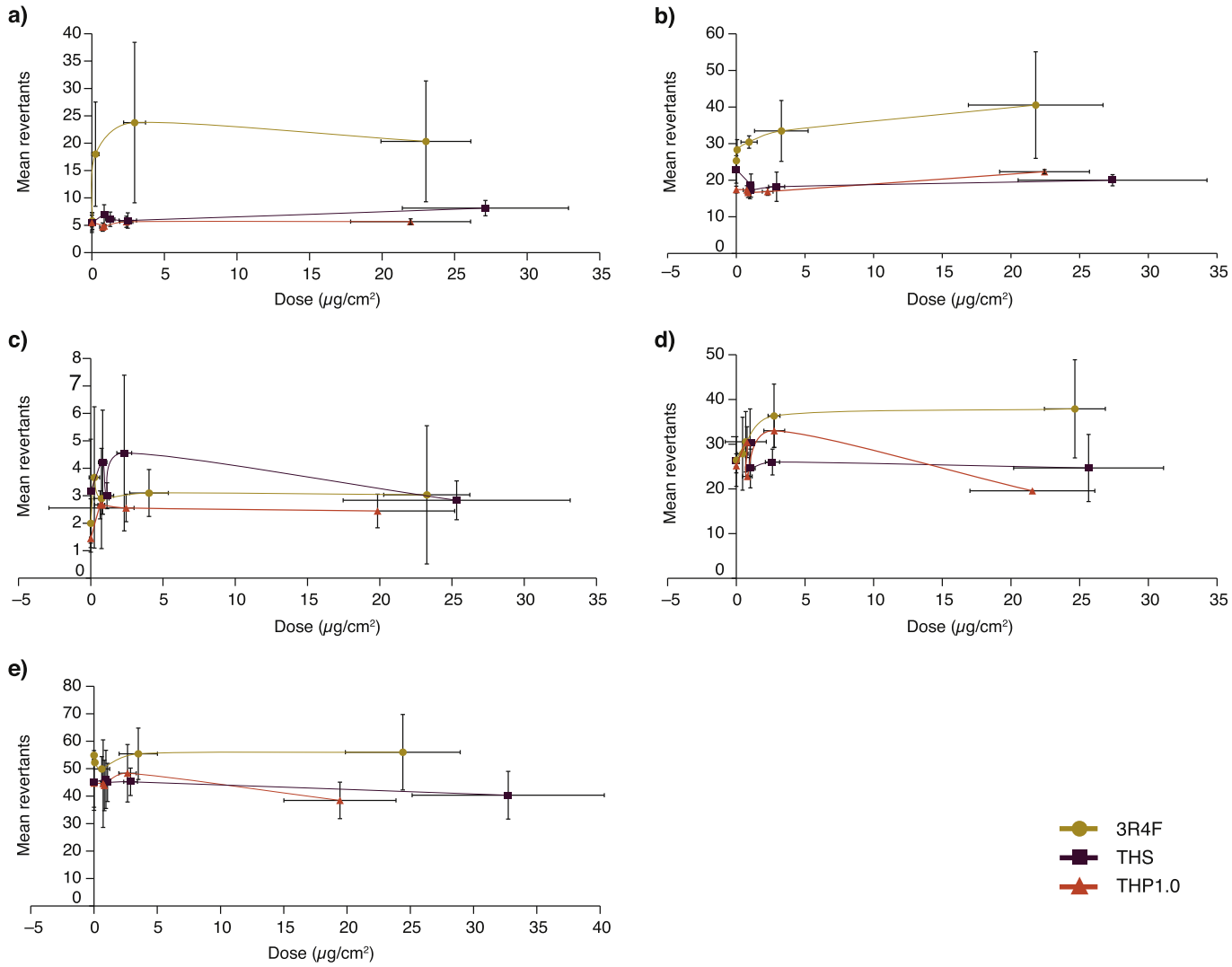

Fig. 5. Reverse mutations using Ames in strains (a) TA98, (b) TA100, (c) TA1535, (d) TA97, and (e) TA102 in the presence of metabolic activation for all three products assessed in response to WA. Mutagenic response can be observed for cigarette smoke in TA98 and TA100 ($p = 0.01$). Both THPs were deemed non-mutagenic under all test conditions.

positive following a 3 h continuous aerosol exposure at any aerosol concentration and with doses up to 40 μg/cm^2 deposited mass. Increases were observed in TA97 following 3R4F cigarette smoke WA exposures but these were not consistent enough between independent experiments to be considered a true positive response.

3.4. Mouse lymphoma assay

Fig. 6 shows the responses in the MLA assay to TPM from all products across the 3 test conditions in the presence and absence of metabolic activation at 3 and 24 h exposures.

TPMs generated from three products were tested in the MLA using L5178Y $tk^{+/-}$ across three test conditions and eight concentrations, 3 h ± S9 and 24 h -S9 [OECD 490]. 3R4F TPM induced significant increases in mutation frequencies when assessed across all three treatment conditions up to toxic concentrations. Increases in mutation frequencies that exceeded the GEF (126 mutants per 10^6 viable cells) were observed at >90 μg/ml following 3 h S9 treatment, at >120 μg/ml following 3 h S9 treatment and at >40 μg/ml following 24 h −S9 treatment, with statistically significant linear trends ($p < 0.001$) observed in all three treatment conditions. 3R4F TPM produced toxicity assessed by relative total growth (RTG) in the 3hr protocol at 100 μg/ml in the absence of S9 and 180 μg/ml in the presence of S9, which gave 12% and 10% RTG, respectively. In the 24hr protocol, the highest concentration analysed, 60 μg/ml, gave an 11% RTG.

TPMs from both THPs did not exceed the GEF in any of the treatment conditions. No significant reproducible or dose responsive increases were observed above the vehicle control in any test condition. No difference was observed between the THPs; neither product differentiated from the control or exceeded the GEF. THS did give a response in the 3 h - S9 protocol at 60 μg/ml, but this response was neither dose responsive nor linear, and the response was deemed not biologically relevant. Both THPs were deemed non-mutagenic under test conditions. RTG values for both THP products did not fall below 63% and 65% in the 24hr -S9 protocol, for THP1.0 and THS respectively at the top concentration tested of 240 μg/ml. Using the 3hr -S9 protocol at the highest dose, 240 μg/mL, RTG values of 75% and 80% for THP1.0 and THS respectively were observed. In the 3hr + S9 protocol 86% and 72% RTG were observed THP1.0 and THS respectively using the highest dose, 240 μg/mL.

3.5. Bhas cell transformation assay

Fig. 7 shows the responses in the Bhas assay to TPM from all products.

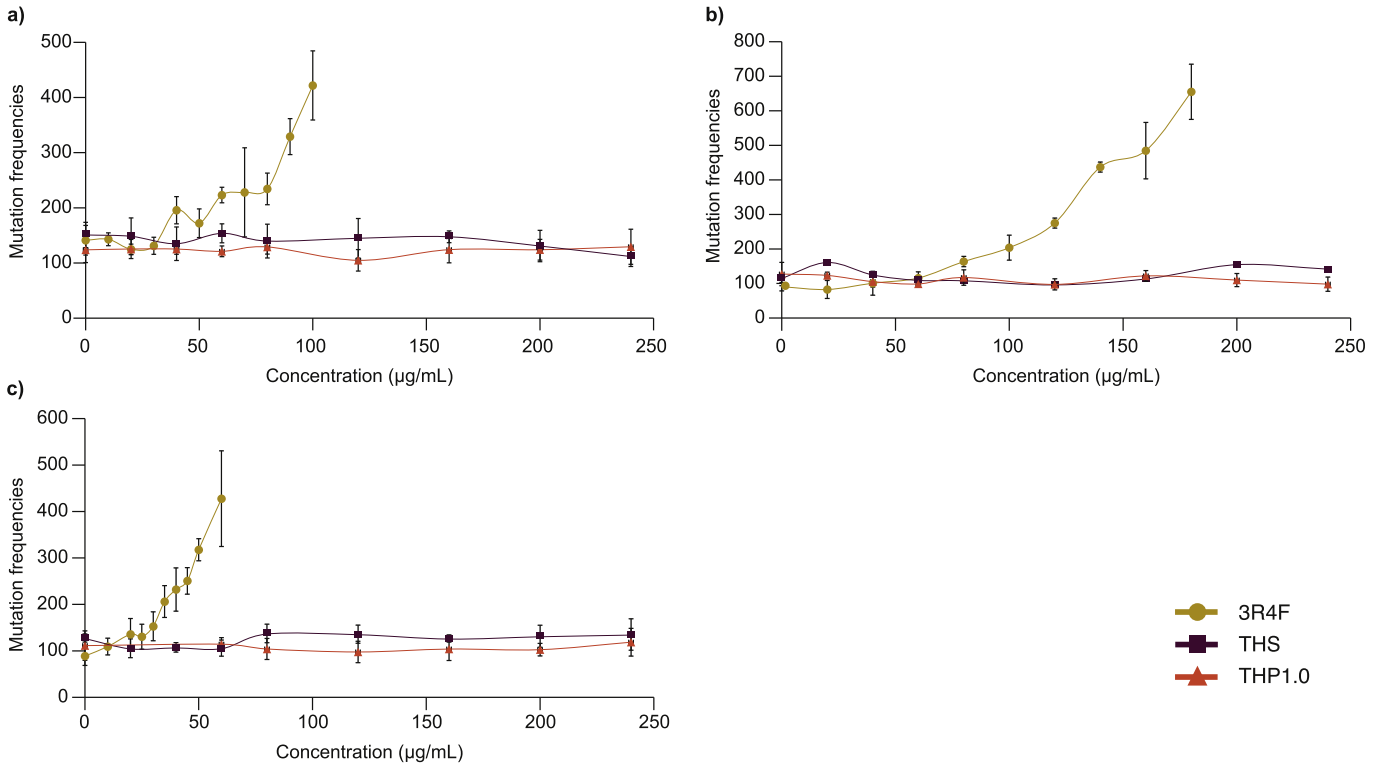

Fig. 6. Mutation frequencies using MLA at (a) 3 h -S9, (b) 3 h + S9, (c) 24 h -S9. Mutagenic response can be observed for 3R4F TPM across all treatment conditions. THP1.0 and THS TPMs were non-mutagenic in all test conditions and did not exceed the global evaluation factor (126 mutants per 10^6 viable cells).

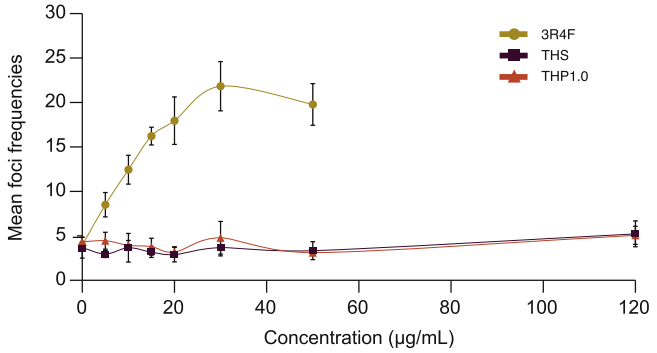

Fig. 7. Effect of TPM from 3R4F reference cigarette and two THPs on *in vitro* promotion in the Bhas 42 cell transformation assay. 3R4F induced a concentration-dependent response and was positive at all concentrations tested, whereas both THPs were negative in this assay.

The Bhas cell transformation assay was employed using the promotion protocol to assess TPM from 3R4F reference cigarette smoke and two commercially available THPs. A preliminary concentration range-finder was performed using concentrations of 1.2–120 µg/ml TPM from the two THPs. 3R4F TPM had previously been shown to exhibit acceptable levels of toxicity at concentrations up to 120 µg/ml in the preliminary toxicity assay protocol, and, therefore, was not tested here. THS was not toxic at the top concentration and THP1.0 induced only 10% toxicity. TPM concentrations of 5–120 µg/ml were selected for the promoter protocol and parallel cytotoxicity assays. 3R4F TPM produced a positive dose response relationship from 5 to 50 µg/ml. At the highest concentration, 120 µg/ml, cell sheets were incomplete due to toxicity. TPM from the 3R4F reference cigarette was classified as positive in the assay (the number of transformed foci increased significantly in at least two consecutive concentrations). 3R4F TPM induced significantly higher numbers of foci than the control treatment at concentrations of 5 µg/ml and above (Dunnett's test, $P < 0.001$). In contrast, the overall activity observed after exposure to TPM from THP1.0 and THS did not differ significantly at any dose from a DMSO control. TPM from the THPs was shown to be less toxic than TPM from the 3R4F in the parallel cell-growth assay. At the top concentration (120 µg/ml), the average viability values were 73.1 ± 7.5% and 71.4 ± 11.0% for the THS and THP1.0 respectively. This was comparable to the top scorable concentration of 3R4F TPM, which had a corresponding average viability of 73.9 ± 6.4%. Positive control TPA treatment resulted in a significant increase in transformed foci in all experiments.

3.6. Summary of results

Table 5 shows a summary and classification of the results obtained for all three products. All raw data relating to each product can be found in supplementary data, broken down by assay type and treatment conditions.

4. Discussion

This study has investigated the genotoxic potential of two THPs (THP1.0 and THS), compared to a 3R4F Kentucky reference product. In this study, the NRU assay was used to assess product cytotoxicity; the bacterial reverse mutation (Ames) assay, the L5178Y $tk^{+/-}$ gene mutation assay (MLA) and the Bhas 42 cell transformation assay were used to assess genotoxicity. MLA was used instead of *in vitro* micronucleus as a measure of mammalian genotoxicity. The Bhas

Table 5
Summary of results.

Treatment condition	NRU[a] TPM	Ames[b] TPM		Ames[c] WA	MLA[d] TPM			Bhas[e] TPM
	−S9	−S9	+S9	+S9	3 h -S9	3 h + S9	24 h -S9	-S9
3R4F[f]	+	+ (TA98, TA100)	+ (TA98, TA100, TA1537)	+ (TA98, TA100)	+	+	+	+
THP1.0	−	−	−	−	−	−	−	−
THS	−	−	−	−	−	−	−	−

Abbreviations: NRU = neutral red uptake assay; TPM = total particulate matter; WA = whole aerosol; MLA = mouse lymphoma assay; + = positive response observed; − = no response observed; THP = tobacco heating product; THS = tobacco heating system.
[a] Tested up to 240 μg/ml over 24 h.
[b] tested up to 2400 μg/plate over 72 h plate incorporation and pre-incubation.
[c] Tested to equivalent dose using quartz crystal microbalance technology (μg/cm^2).
[d] tested up to 240 μg/ml with and without metabolic activation over three treatment conditions (3 h with and without S9 and 24 h without S9).
[e] Tested up to 120 μg/ml for 10 days.
[f] Affected *Salmonella typhimurium* tester strains are shown in brackets.

42 cell transformation assay has an OECD guidance document (OECD, 2016) and was used to complement the testing strategy in predicting carcinogenic potential, using the promotion protocol. In addition to TPM test matrices, WA was also assessed in the Ames assay. Collectively, the results from all *in vitro* techniques were analysed in a weight of evidence approach.

Acute cytotoxicity was assessed in this study using the ICCVAM NRU protocol in BALB/c 3T3 mouse fibroblasts (ICCVAM, 2006). 3R4F TPM was shown to be cytotoxic in a range of 20–140 μg/ml. An IC_{50} was achieved at 81.2 μg/ml. Neither the THP1.0 or THS showed a decrease in viability beyond that of the control and an IC_{50} for these products could not be defined even at the highest achievable dose of 240 μg/ml. Therefore, under the test conditions in this study THP1.0 and THS were classified as non-cytotoxic after failing to achieve an IC_{50}, in line with the protocol guidelines.

S. typhimurium strains TA98, TA100, TA97, TA1535, TA1537 and TA102 were used to assess the mutagenic potential of TPMs from THPs and 3R4F (OECD, 1997). 3R4F TPM demonstrated positive mutagenic responses in tester strains TA98, TA100 and TA1537 in the presence of metabolic activation throughout the dose range from 50 to 2400 μg/plate. In the absence of metabolic activation, strains TA98 and TA100 were found to positively mutated in response to 3R4F cigarette smoke TPM. Responses in the absence of metabolic activation were observed in the same range, but in general were lower in revertant numbers than those observed in the presence of S9. 3R4F cigarette smoke TPM did not induce reverse mutations in any of the test conditions in strains TA1535 and TA102 in the presence or absence of metabolic activation. THP1.0 and THS did not induce any mutations above the untreated background controls in any strain either in the presence or absence of metabolic activation, even at the top dose of 2400 μg/plate.

The observed responses in the presence and absence of metabolic activation with a reference 3R4F cigarette are consistent with previously reported TPM findings, in that TA98, TA100 and TA1537 were all considered positive in the presence of metabolic activation, whereas TA1535 and TA102 were negative (Combes et al., 2012, 2013; Schaller et al., 2016). The Ames assay has been previously used to assess the mutagenic activity of THPs, (Schaller et al., 2016), with similar reported responses. A study by Foy et al., (2004), assessed the mutagenic activity of an early version of tobacco heating technology, Eclipse, in tester strain TA98 and demonstrated that mutagenicity was significantly lower than for conventional tobacco products. Both studies have concluded the same as this study, in that tobacco heating technology is clearly less mutagenic compared to tobacco smoke. To complement testing approaches, whole aerosol techniques were employed using a modified Ames methodology in strains TA98, TA100, TA1535, TA97 and TA102 in the presence of metabolic activation. This approach enabled the assessment of freshly generated aerosol, which offers the advantages that the test aerosol is not trapped or fundamentally altered and is a more representative test matrix for human use (Thorne and Adamson 2013; Thorne et al., 2016; Xiang et al., 2014). Furthermore, aerosol approaches capture the interactions between the vapour phase and particulate phase constituents, where trapping techniques might omit one or both. Supporting findings from TPM assessments, WA from 3R4F cigarette smoke was considered mutagenic in strains TA98 and TA100 observed from 3 μg/cm^2. In contrast, the THPs investigated, THP1.0 and THS were negative in all strains when assessed up to 20–40 μg/cm^2. In another study, Ishikawa et al. (2016) assessed a prototype THP in an aerosol Ames assay under comparable conditions and concluded that it had a lower mutagenic activity index compared to traditional tobacco smoke. Breheny et al. (2017b) also report a lower mutagenic potential of WA from a THP compared to 3R4F whole smoke, using the modified Ames assay. These results, using a similar modified Ames assay are consistent with the findings from this study, in that the THP1.0 and THS were non-mutagenic compared to the mutagenic nature of a traditional combustible reference product.

This study has further assessed mutations in MLA assay using L5178Y cells with the $tk^{+/-}$ mutation. TPM from all products were assessed under three test conditions, 3 h with and without metabolic activation and 24 h without metabolic activation. In all three test conditions, 3R4F TPM produced a positive linear dose–response relationship, from 35 to 180 μg/ml (depending on treatment condition). Conversely, neither THP1.0 or THS produced as response exceeding the GEF at any doses assessed in the study. Schaller et al. (2016) also conducted MLA testing on the THS and demonstrated a positive response in the presence of metabolic activation (3 h), and varied response in its absence (3 and 24 h). In our study, neither device produced a positive response under the assessment conditions irrespective of S9 treatment. Noticeably, the Schaller et al. (2016) study found mutagenicity in the range of 10–20% RTG. Our study did not induce the same levels of toxicity due to TPM dose-matching against a combustible reference product. Additional testing at higher doses may be required to fully assess the mutagenic capacity of these devices to avoid reporting false negatives. However, compared to 3R4F TPM at equivalent doses, neither THP1.0 or THS significantly induced mutation frequencies above the GEF under any test condition. Irrespective of toxicity, compared to 3R4F TPM, THS and THP1.0 were not only significantly reduced but also deemed non-mutagenic.

Finally, to support a weight of evidence approach, the Bhas

42 cell transformation assay, which detects tumour-promoting activity, was also employed in this study. The Bhas assay is not currently recognised in any regulatory approved testing strategy, such as those guidelines issued by ICH, COM and Health Canada. However, an OECD guidance document has recently been published (OECD, 2016). Therefore, in this study, to complement testing approaches the Bhas 42 cell transformation assay was employed, using the promotion protocol for predicting carcinogenic potential. This assay has been previously applied with high sensitivity and good precision to cigarette smoke (Weisensee et al., 2013) and THPs (Breheny et al., 2017a). TPM from a standard research cigarette was found to induce a dose-dependent increase in type III foci, with up to a 20-fold increase in focus formation at moderately toxic concentrations (5–60 μg/ml) relative to the control (Weisensee et al., 2013). In addition, a study that assessed the tumour-promoting activity of a hybrid tobacco heating product relative to 3R4F cigarettes showed that 3R4F TPM induced significantly higher numbers of foci than control treatment at concentrations of 15 μg/ml and above, whereas the hybrid-THP was negative at all concentrations tested (Breheny et al., 2017b). In the present study, exposure to TPM from 3R4F cigarettes elicited a concentration-dependent response and was deemed to be tumour-promoting. Neither THP1.0 or THS produced a positive response in the assay under test conditions with up to 120 μg/ml test article. The top concentration of TPM was limited by the amount of DMSO solvent the assay system can be exposed to. Traditional TPM extracts can be as high as 1% DMSO at top doses of 240 μg/ml. Given the lack of historical data on the Bhas 42 cell transformation assay, the top dose was limited to the guideline limits of 0.5%, which equated to top dose of 120 μg/ml. Future studies will assess higher DMSO concentrations on the transformation of foci to achieve a more concentrated top dose.

All endpoints assessed confirm reduced *in vitro* biological and toxicological effects with THP1.0 compared to 3R4F reference cigarette smoke, in fact both THPs (THP1.0 and THS) failed to elicit a biological response under any of the conditions tested. When analysing the levels of nine key toxicants compared to 3R4F cigarette smoke, THP1.0 showed significant reductions (Eaton et al., 2017; Forster et al., 2017). For example, the CO level, which is related to combustion processes, was reduced. Acetaldehyde and formaldehyde were considerably lower, which are linked to the decomposition of sugars, either added to the leaf or naturally present in the tobacco leaf (Polat et al., 2016a; Polat et al., 2016b; Roemer et al., 2012; Várhegyi et al., 2010). Volatile organic compounds, such as acrolein, benzene and 1,3-butadiene were reduced to or below the level of detection. These chemicals have multiple formation routes, but are typically formed by pyrolysis and incomplete combustion of tobacco (Piadé et al., 2013). Benzo[a] pyrene, *N*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are found in conventional cured tobacco leaf and leaf-curing processes and were also significantly reduced (Eaton et al., 2017). Benzo[a]pyrene, a polycyclic aromatic hydrocarbon, can also form as part of incomplete combustion of the organic plant material and is usually formed at 300–600 °C. Pyrosynthesis of NNN and NNK in a lit cigarette is thought to involve reactions occurring above 240 °C (Rodgman and Green, 2014) and, therefore, the reductions in the levels of these chemicals is not surprising considering THP1.0 does not exceed 250 °C (Eaton et al., 2017). Reductions in these constituents in the emissions from the THPs may translate to the reduced *in vitro* toxicity profile observed compared to 3R4F cigarette smoke.

5. Conclusions

Overall, in a weight of evidence based approach, 3R4F reference cigarette smoke was deemed significantly positive in all assays. At equivalent doses to cigarette smoke, the commercially available THP1.0 and THS failed to elicit a positive response in any of the endpoints assessed, despite appropriate positive and negative control responses validating assessments. Both THPs produced an equivalent negative response in all test systems and protocols, suggesting at least parity between the devices. Future testing strategies must investigate these NGPs at doses significantly exceeding that of tobacco-smoke exposure to ensure that the results generated here are a reliable reflection of the actual *in vitro* biological activity. However, this study has clearly demonstrated that compared to cigarette smoke at equivalent doses in TPM and WA techniques, THPs demonstrate significantly reduced *in vitro* toxicological activity.

Declaration of interest

The authors are employees of British American Tobacco. All experimental work was funded by British American Tobacco. Covance Laboratories (Harrogate, UK) generated TPM and conducted Ames, MLA and NRU studies. BioReliance (USA) conducted Bhas studies.

Authors contributions

David Thorne, Damien Breheny and Marianna Gaca designed the studies and wrote the manuscript. Christopher Proctor oversaw the research programme. All authors approved the final version.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.yrtph.2017.08.017.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.yrtph.2017.08.017.

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