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Genotoxicity evaluation of tobacco and nicotine delivery products: Part One. Mouse lymphoma assay

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ABSTRACT

Conduct of the mouse lymphoma assay (MLA) is underpinned by Organisation for Economic Co-operation and Development (OECD) Test Guideline 490 and International Conference on Harmonisation S2(R1) guidance and is a recognised *in vitro* genotoxicity test battery assay. It has been used on a limited number of occasions for the assessment of some tobacco and nicotine products, such as e-cigarettes and tobacco heating products (THP). The aim of this study was to assess the suitability of the MLA for genotoxicity testing with a variety of tobacco and nicotine products.

Total particulate matter (TPM) from a 3R4F cigarette was compared against a commercial electronic cigarette liquid (e-liquid), electronic cigarette (e-cigarette) aerosol matter captured from the same e-liquid, and TPM from a commercial THP. Treatment conditions included 3 h exposures with and without metabolic activation and a longer 24 h exposure without metabolic activation (-S9) at concentrations up to 500 μg/mL.

Under all treatment conditions, 3R4F produced a clear positive response with regard to induction of mutation. In contrast, no marked induction of mutation was observed for the e-liquid, e-cigarette aerosol or THP. Additionally, data are presented as a function of nicotine equivalents for comparisons between these different tobacco products and test matrices.

1. Introduction

Cigarette smoking is a major risk factor for cardiovascular disease, chronic obstructive pulmonary disorder and cancer. Next generation tobacco and nicotine products (NGPs), which include tobacco heating products (THPs) and electronic cigarettes (e-cigarettes, or alternatively termed Electronic Nicotine Delivery Systems (ENDS)), have evolved significantly over the last few years and are gaining growing consumer acceptability. Recent research suggests that both THPs and e-cigarettes yield fewer toxicants and at lower levels in their respective aerosols compared to that of 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) As yet, it is unclear if the reduction in chemicals and toxicants yielded by these products (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) will translate into reduced risk to public health. Certainly, *in vitro, in vivo* and clinical data suggests 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). Recent clinical studies have demonstrated significantly reduced levels of biomarkers of exposure in e-cigarette and THP users' urine compared to that of regular smokers (Gale et al., 2018; Hecht et al., 2015), therefore, implying that reduced exposure to such compounds is an outcome of e-cigarette and THP use as opposed to use of combustible tobacco.

THPs operate by primarily heating a specifically designed tobacco-rod up to temperatures significantly lower than those observed in combustible cigarettes. Heating temperatures of THPs are typically much lower than the temperature within the coal of a burning cigarette (Baker, 1974; Forster et al., 2018; Smith et al., 2016). The result is a THP aerosol that is less complex than tobacco smoke, with a significantly reduced chemical profile and lower levels of measurable toxicants (Borgerding et al., 1997; Eaton et al., 2018; Forster et al.,

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2018; Smith et al., 2016; Schaller et al., 2016).

Electronic cigarette liquids (e-liquids) are typically formulated with pharmaceutical grade propylene glycol and/or vegetable glycerine mixtures often (but not always) containing nicotine and flavourants. The e-cigarette is typically comprised of a coil and wick, battery, microprocessor and e-liquid storage device (tank or closed cartomiser, which is a cartridge containing e-liquid). Due to the simplicity of the starting e-liquid the resulting aerosol is equally simple. The e-cigarette coil is heated to a range of approximately 140–230 °C, depending on the device (Chen et al., 2018; Zhao et al., 2016). The maximum temperature is dependent on the composition of the e-liquid (PG/VG/H_2O ratios), how the device is used and power setting. Some current advances in e-cigarette technologies now allow the device to automatically control the power setting and the maximum achievable coil temperature to minimise potential misuse and dry-wicking scenarios, which can ultimately result in the formation of toxicants (Farsalinos et al., 2015). Chemical studies of e-cigarette aerosols have shown that most commonly characterized combustible cigarette toxicants are below the limit of detection with current methods (Margham et al., 2016), raising questions of their existence within the e-cigarette aerosol. On a puff-by-puff basis, studies have shown that both e-cigarette and THP aerosols show significant reductions in key toxicants, which include metals, nitrosamines and carbonyl compounds, when compared to a 3R4F reference cigarette (Borgerding et al., 1997; Forster et al., 2018; Margham et al., 2016; Roemer et al., 2012). In addition, some toxicants such as carbon monoxide, hydrogen cyanide, benzene and acrylonitrile amongst others are not detectable in NGP aerosols (Forster et al., 2018; Margham et al., 2016; Roemer et al., 2012). Public Health England have stated that e-cigarettes could be up to 95% safer compared to use of traditional cigarettes (McNeill et al., 2015).

There are currently no international guidelines on what pre-clinical *in vitro* tests are most appropriate for assessing NGPs. As part of a pre-clinical assessment strategy, a battery of *in vitro* tests may be used for the assessment of the toxicological potential of NGPs. Previous studies have reported various techniques, which include classical genotoxicity techniques, 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 the classical toxicological approaches, clear and complementary international guidelines exist, which recommend various *in vitro* mutagenicity, genotoxicity and cytotoxicity assays. These include guidelines developed by the International Conference on Harmonisation S2(R1) (2011), the UK Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (2011), Health Canada (2005) and the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) (2004). In summary, these guidelines call for the use of multiple assays to assess test articles for a variety of toxicological endpoints. For example, the bacterial mutagenicity assay (Ames bacterial reverse mutation assay), the *in vitro* micronucleus assay (IVMN) and/or the mouse lymphoma assay (MLA), combined with an acute cytotoxicity assay are commonly recommended. Depending on guidelines, the chromosome aberration assay may also be used (Cooperation Centre for Scientific Research Relative to Tobacco, 2004; International Conference on Harmonisation, 2011). The mammalian IVMN and MLA are accepted equally from a regulatory perspective according to ICH guidelines; choice between the two assays may be informed by the availability of historical data, prior use and personal laboratory preference.

The MLA *tk* $^{\pm}$ assay is one of the most widely used and accepted mammalian gene mutation tests for screening and regulatory testing that is also sensitive to the action of chromosome-breaking agents and other genotoxic changes. The assay works via the mutation of the *tk* (thymidine kinase) locus in mouse lymphoma cells and can detect both gene mutations and chromosome aberrations. The assay utilises a forward mutation occurring at the wild-type thymidine kinase (*tk*) gene locus, resulting in a change from *tk* $^{\pm}$ to $tk^{-/-}$ which causes the loss of thymidine kinase (TK) enzyme activity. Deficiency in the enzyme is not lethal as cells can survive using *de novo* synthesis of thymidine. The cells used in the MLA assay (L5178Y 3.7.2C cells) are heterozygous at the *tk* locus ($tk^{+/-}$), therefore a single mutational event in the tk^{+} gene on chromosome 11b leads to forward mutation to the $tk^{-/-}$ genotype with little or no TK activity. Loss of functional tk^{+} expression renders cells resistant to toxic thymidine analogues that can be phosphorylated by TK, e.g. trifluorothymidine. Accordingly, trifluorothymidine can be used to select $tk^{-/-}$ mutant colonies in a background of *tk* $^{\pm}$ cells (Law, 1952). Mutation frequency (MF) is estimated by comparing the cloning efficiency of the cells in culture medium without the selective agent. Mutagenic activity is then determined by treating cultures with different concentrations of a test article and examining the potential for concentration-related increases in MF.

A wide variety of mutagenic events can lead to trifluorothymidine resistance, including small mutations within the tk^{+} gene (gene mutation) or larger clastogenic (chromosome breakage) events within and beyond the tk^{+} gene which may alter the structure of chromosome 11b, such as deletions, translocations and mitotic recombinations. It has been proposed that the assay can detect whole chromosome loss due to chromosomal nondisjunction or aneuploidy (Fischer, 1958), but there is considerable debate regarding whether the assay can reliably detect *in vitro* aneugenicity. Furthermore, mutant $tk^{-/-}$ colonies can have slow or wild-type growth rates and the difference in mutant colony growth has been attributed to different mechanisms of DNA damage. Chromosomal mutations extending beyond the *tk* gene produce small, slow-growing mutant colonies, while intragenic mutations tend to produce large clones akin to those observed for wild-type cells (Combes et al., 1995; Fellows et al., 2011). Therefore, mutant colony size can be potentially used as an indicator as to the mode of action of the test chemical (Blazak et al., 1986, 1989).

In this study, particulate-based test articles (TPM) generated from a commercially available THP and e-cigarette were compared to cigarette smoke TPM, which was generated in a comparable manner. The e-liquid, unaerosolized, from the same e-cigarette was also compared to TPMs from cigarette smoke, THP and the e-cigarette. Finally, NGP test articles were compared to cigarette smoke TPM based on a nicotine dose equivalence to demonstrate consistent dosing between tobacco product types. This manuscript describes the use of the MLA assay for the assessment of NGPs as part of a testing paradigm, but comprises Part One of a two-part series on the genotoxicity evaluation of the same NGPs. Part Two focuses on the use of the IVMN assay for the assessment of NGPs using the same test articles (Thorne et al., 2018b).

2. Materials and methods

Testing was conducted in accordance with OECD Test Guideline 490 (OECD, 2015). All chemicals and reagents were obtained from Sigma-Aldrich UK, unless otherwise stated.

2.1. Test articles

Three products were assessed: a reference cigarette and a commercially available THP and e-cigarette. These were evaluated in the form of four separate test articles. That is, TPM was collected from the reference cigarette, THP and e-cigarette aerosols, while the e-liquid from the same e-cigarette was also assessed directly. This was to determine if the aerosolisation process imparted any differential genotoxicity on the e-liquid in comparison to that from the liquid itself.

3R4F Kentucky Reference (University of Kentucky, USA) TPM was generated using 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 duration and 100% vent blocking using a bell shaped puff profile (Health Canada,

1999). THP TPMs were generated with a RM200a smoking machine (Borgwaldt, Hamburg, Germany) using a modified HCI puffing regimen, described as a 55 mL puff volume every 30 s with a 2 s puff duration, with no vent blocking, again puffed using a bell shaped smoking profile. Vent blocking is not always achievable with THP consumables, so in this study vent blocking was not employed. E-cigarette TPM was generated using a RM200a smoking machine under the CORESTA recommended method 81 puffing regimen, described as a 55 mL puff volume every 30 s with a 3 s puff duration, and a square wave puff profile to allow product actuation (Cooperation Centre for Scientific Research Relative to Tobacco, 2015).

For 3R4F cigarettes, THPs and e-cigarettes, 10, 12 and 60 puffs were used per consumable/cartomisers, respectively. Fresh consumables 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 compared to THP and e-cigarette TPM collection to avoid cross contamination. Recent studies (Adamson et al., 2016, 2017) suggest that different smoking machines used for *in vitro* studies performed the same under a given set of smoking conditions if appropriately maintained. Therefore, the split in machine smoking across products was deemed preferable to cross-category-contamination.

Table 1 shows a breakdown of the products, specifications, regimens used and parameters for test article generation.

TPM from the reference cigarette and NGPs was collected onto 44 mm Cambridge filter pads (Whatman, Maidstone, UK). Pads were weighed before and after test article 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 and prepared in DMSO prior to use.

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 pad was also collected. Partner and quality control pads were analysed for nicotine, water and glycerol content using gas chromatography techniques (total combustible gas and flame ionisation detector).

A summary of the test article TPM characterisation is shown in Table 2.

Test articles were prepared at 100x the required final concentrations approximately 3 h prior to treatment and diluted in DMSO to provide lower concentrations for testing. The test articles were added to the system at a final concentration of 1% v/v (100-fold dilution).

2.2. Controls

Cultures treated with DMSO only were used as vehicle controls. Concurrent positive control chemicals methyl methane sulphonate in the absence of S9 and benzo[*a*]pyrene in the presence of S9 were included in each experiment.

2.3. Metabolic activation system

The mammalian liver post-mitochondrial fraction (S9) used for metabolic activation was obtained from Molecular Toxicology Incorporated (MOLTOX, USA) where it is prepared from male Sprague Dawley rats induced with Aroclor 1254. The batches of MOLTOX™ S9 were stored frozen in aliquots at < -50 °C prior to use. Each batch was checked by the manufacturer for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufin-O-dealkylase activities).

The S9 mix contained G6P (180 mg/mL), NADP (25 mg/mL), potassium chloride (150 mM) and rat liver S9, mixed in the ratio 1:1:1:2. For all cultures treated in the presence of S9, an aliquot of the mix was added to each cell culture to achieve the required final concentration of test article in a final volume of 20 mL. The final concentration of the liver homogenate in the test system was 2%.

2.4. MLA

Mouse lymphoma (L5178Y $tk^{+/-}$) cells originated from Dr. Donald Clive, Burroughs Wellcome Co. Test articles were assessed under three test conditions, 3 h with and without S9 and 24 h without S9. Cell cultures were maintained in Roswell Park Memorial Institute medium cell culture media (RPMI-1640), supplemented with 10% v/v heat-inactivated horse serum and penicillin and streptomycin (RPMI 10) at 37 °C ± 1 °C.

Table 1
Product specifications.

Category	Cigarette	NGPs	
Sub-category	Combustible Cigarette	Tobacco Heating Product (THP)	Electronic Nicotine Delivery System (ENDS) or electronic cigarettes (e-cigarettes)
Product description	Reference	Commercially available products	
Product design	Combustible cigarette	Intelligently controlled heating system. Button activated to initiate heating.	Closed modular puff activated system with interchangeable cartomisers.
Aerosol generation	Combustion, distillation and condensation	Distillation and condensation	Evaporation, Supersaturation, Homogenous nucleation, coagulation and condensation
Test article	TPM	TPM	e-liquid & TPM
Top Dose (TPM or e-liquid: µg/mL)	200	500	500
Puffing Regimen	HCI	HCI_m	CRM81
Laboratory test conditions	ISO-3402	ISO-3402	ISO-3402
Puffs per product	10	12	60
Blend/Consumable/E-liquid	US blend	Tobacco flavour	Tobacco flavour
Nicotine content[a]	9.0 mg/consumable[b]	1.0 mg/consumable[c]	1.8 mg/mL[d]

TPM = total particulate matter, THP = tobacco heating product, ENDS = electronic nicotine delivery system, HCI = Health Canada intense puffing regimen, HCI_m = Health Canada intense modified puffing regimen, CRM81 = CORESTA recommended puffing regimen, ISO = international standards organisation.

[a] = Cigarette ISO and THP HCI nicotine yields.

[b] Roemer et al. (2012).

[c] Schaller et al. (2016).

[d] From product packaging.

Table 2
Total Particulate Matter (TPM) characterisation.

Product	mg/consumable (± SD)					
	TPM	Nicotine	Water	NFDPM	Glycerol	Propylene glycol
3R4F	37.8 (1.8)	1.9 (0.04)	11.7 (0.8)	24.3 (0.7)	N/A	N/A
THP	31.8 (0.3)	1.8 (0.1)	16.7 (0.4)	106.9 (3.9)	72.2 (2.4)	31.6 (0.7)
e-cigarette	125.4 (3.9)	1.1 (0.01)	21.0 (0.8)	4.3 (0.1)	4.3 (0.1)	0.6 (0.05)

Data are mean (± standard deviation) of 3 independent TPM collections.
Products puffed under HCI, HCI_m or CRM 81 for cigarettes, THP and e-cigarette respectively (see Table 1).
Abbreviation: NFDPM = nicotine-free dry particulate matter, N/A = not assessed.

For exposure, at least 1×10^7 cells or 4×10^6 cells for the 3 h or 24 h exposures, respectively, were placed in centrifuge tubes (3 h) or culture flasks (24 h) and test article, positive control or vehicle control were added. Following the appropriate exposure periods, cells were centrifuged (200 *g*) washed and transferred to tissue culture flasks for the 2-day expression period, during which the *tk* $^{-/-}$ mutation would be expressed. Cultures were maintained for 48 h from the end of the respective treatment incubation period to assess L5178Y *tk* $^{-/-}$ expression. At the end of the expression period, cells were plated for viability and mutation. Viability was assessed by plating 2 × 96-well microtitre plates (192 wells with an average of 1.6 cells/well). Mutation was assessed by plating 4 × 96-well microtitre plates (384 wells with 2000 cells/well) in medium containing trifluorothymidine at a final concentration of 3 μg/mL. For each assessment, plates were incubated for 1–2 weeks at 37 °C ± 1 °C in a humidified atmosphere with 5% CO_2. The total number of wells and numbers of wells containing large and small colonies were scored for all vehicle and positive control cultures, and where the MF of any test article treatments exceeded the MF of the vehicle control plus the global evaluation factor (GEF). For microwell assays, the GEF is defined as 126 mutants per 10^6 viable cells.

2.5. Analysis of results

Suspension Growth (SG) was measured as growth in suspension during treatment and through the expression period. Relative suspension growth was measured relative to the mean vehicle control SG. Viability measured the ability of the cells to clone, i.e. Cloning Efficiency. Relative Total Growth (RTG) measured cytotoxicity, relative to the vehicle control, taking into account all cell growth, cell loss during the treatment period and the 2 day expression period (relative suspension growth) and the ability to clone 2 days after treatment (viability).

MF was calculated from the number of trifluorothymidine-resistant mutants expressed per 10^6 viable cells. Small and large colony MF values were calculated in an identical manner, using the relevant number of empty wells in the microtitre plates for small and large colonies, as appropriate. The relevance of increases in mutant frequencies (total wells with clones), by comparison with concurrent controls and the global evaluation factor (GEF), was assessed according to the recommendations of Moore et al. (2006). Linear regression was performed on ranked MF against ranked concentration to test for a linear trend. The linear trend was performed on the overall MF per concentration level, not individual replicates (i.e. one MF value per concentration). The test for linear trend is one-tailed, therefore negative trend was considered not relevant. The positive control was excluded from the test for linear trend.

Each test article was tested in two replicate experiments. The response in each experiment was evaluated, followed by an overall assessment of mutagenic response across both experiments.

2.6. Data analysis

The data from the samples were statistically compared against each other only if they gave a positive response in both experiments. Data were analysed using log-transformed MF. Statistical comparisons were made according to previously described methodologies (Scott et al., 2013). Briefly, for each test article, the response was defined as the slope of the linear portion of the concentration-response curve, for each combination of treatment condition and experiment. The linear portion of the concentration-response curve was determined by fitting a linear model including a quadratic term for concentration. The portion of the concentration-response curve was deemed to be linear when the quadratic term for concentration was non-significant ($p \geq 0.01$).

Assays were considered valid if the following criteria were met: 1) the mean MF in the control cultures fell within the laboratory's historical range (i.e., 50 to 170 mutants/10^6 viable cells); 2) at least one positive control showed either an absolute increase in mean total MF of at least 300×10^{-6} (at least 40% of this should be in the small colony MF), or an increase in small colony MF of at least 150×10^6 above the concurrent vehicle control; 3) the RTG for the positive controls were > 10%; 4) the mean cloning efficiency of the vehicle controls from the mutation experiments was between 65% and 120%; and finally, 5) the mean suspension growth of the vehicle controls from the mutation experiments was between 8 and 32 following 3 h treatments or between 32 and 180 following 24 h treatments.

The test article was considered as mutagenic in an experiment if the following evaluation criteria were met: 1) the MF of any test concentration exceeded the sum of the vehicle control MF plus GEF; 2) the linear trend test was statistically significant and reproducible across two independent experiments. The test article was considered positive if both criteria 1 and 2 were met; the test article was considered negative if neither criterion 1 and 2 were met.

Data are presented as induced MF, with background (mean vehicle control MF) subtractions.

3. Results

All raw data including RTG, MF and induced MF can be found in supplementary material, broken down by experiment with statistical observations (Tables S1–S5). Each experiment was conducted on two independent occasions.

3.1. Controls

Vehicle and positive control treatments were included in every experiment and under each test condition. MF values in the vehicle control cultures generally fell within ranges of between 50 and 170 mutants per 10^6 viable cells and were considered acceptable. Clear increases in MF were induced by the positive control chemicals methyl methane sulphonate and benzo[*a*]pyrene in the absence and presence of S9, respectively (data not shown).

3.2. RTG and MF

Total particulate matter (TPM) samples of reference cigarette (3R4F) smoke, THP and e-cigarette (e-liquid and TPM) were assayed for the ability to induce mutation at the wild-type *tk* locus (5-trifluorothymidine resistance) in mouse lymphoma cells using a fluctuation protocol. TPM was assessed across three test conditions and up to ten test concentrations, defined via historical knowledge or a pre-range finder experiment for cytotoxicity (data not shown). In each case the test article was tested up to the maximum achievable concentration defined by either cytotoxicity (relative total growth/RTG) or solvent concentration (DMSO). 3R4F TPM was tested up to 200 μg/mL, whereas TPM from the NGPs (THP and e-cigarette) were tested up to 500 μg/mL, achieved by generating a more concentrated stock solution and normalising for DMSO content (not exceeding 1%).

When tested up to toxic concentrations of 3R4F TPM, increases in MF which exceeded the GEF of 126 mutants per 10^6 viable cells (compared to concurrent controls) were observed at 60 μg/mL and above for the 3 h treatment in the absence of S9, at 120 μg/mL and above for the 3 h treatment in the presence of S9, and at 20 μg/mL and above for the 24 h treatment in the absence of S9. Responses were observed in two independent experiments, demonstrating statistically significant linear trends ($p \leq 0.001$). 3R4F TPM produced toxicity as assessed by RTG in all treatment conditions (3 h ± S9 and 24 h -S9), at varying concentrations depending on the exposure time and addition of S9. The 3 h treatment saw RTG values of 15 and 12% for 100 and 200 μg/mL in the absence and presence of S9, respectively, in Experiment 1 and corresponding RTG values of 14 and 12% at 100 and 200 μg/mL in the absence and presence of S9, respectively, in Experiment 2. Under 24 h exposure conditions in the absence of S9, a concentration of 50 μg/mL gave 9% RTG in Experiment 1. Steep concentration-related toxicity was observed between 40 and 50 μg/mL, giving 31% and 9% RTG, respectively. In Experiment 2, the maximum concentration analysed (65 μg/mL) gave 11% RTG, demonstrating comparable results between experiments.

When THP TPM was tested up to the maximum achievable concentration of 500 μg/mL in two experiments, no reproducible increases in MF were observed under any treatment condition. At the maximum concentration of THP exposures, RTG values between experiments were 75 and 63% for 3 h -S9, 79 and 68% for 3 h + S9 and 45 and 50% for 24 h -S9 in Experiments 1 and 2, respectively. RTG did not fall below 40% in the presence or absence of S9.

E-cigarette TPM, when tested up to 500 μg/mL, showed no increases in MF above the GEF under any treatment condition in either experiment. RTG did not fall below 80% in any treatment condition up to a maximum concentration of 500 μg/mL. E-cigarette e-liquid showed a similar trend with no observed increases in MF above the GEF under any treatment condition in either experiment. The RTG values observed for e-liquid concentrations were even higher than that of THP and e-cigarette TPM, with RTG values very similar to those observed in the vehicle controls under each treatment condition and experiment.

Fig. 1 shows the induced MF values for cigarette, THP and e-cigarette (TPM and e-liquid) and is a representative dataset taken from Experiment 1. The full data set can be found in supplementary materials (Supplementary Tables S1–S4).

3.3. Nicotine equivalents

Traditional TPM appproaches display the data as a function of particulate concentration (TPM μg/mL). However, when assessing NGPs where the aerosols are chemically and compositionally very different, a more relevant comparison may be required. In this case, data were normalised for nicotine in each test matrix and presented as a function of nicotine to illustrate responses compared to cigarette smoke.

Fig. 2 shows the induced MFs for cigarette, THP and e-cigarette (TPM and e-liquid) and is a representative dataset taken from Experiment 1. MF values have been presented as a function of nicotine concentration calculated from the stock (Table S5 in supplementary materials). The data shows that on all occasions at the maximum concentration of cigarette smoke and where a positive response was observed, that for the NGPs (THP and e-cigarette), nicotine equivalence has been approximately reached. In the case of THPs, the nicotine concentration delivered far exceeded that of cigarette smoke.

Table 3 shows the nicotine dose delivered to the cells and the corresponding TPM concentration. Under each treatment condition, the maximum concentration of cigarette smoke TPM is reported with a corresponding nicotine concentration. For THP and e-cigarette (TPM and e-liquid), the closest nicotine concentration reaching equivalence is reported with its corresponding TPM/e-liquid concentration. Under all treatment conditions, nicotine equivalence was reached well in advance of the maximum cigarette smoke concentration, except for the 3 h + S9 treatment, where equivalence was reached at the maximum concentration of e-cigarette test articles (TPM and e-liquid) of 500 μg/mL and at a concentration of 300 μg/mL for THP TPM. Table S5 in supplementary materials details the ranges and calculated nicotine concentrations for each concentration, based on the stock concentration.

4. Summary of results

Overall results are summarised in Table 4.

5. Discussion

The aim of this study was to compare the genotoxic potential of an e-cigarette and THP to a reference cigarette (3R4F) using the mouse lymphoma assay at the *tk* locus. Three test conditions were assessed (3 h ± S9 and 24 h -S9), with up to 10 concentrations per treatment condition across two independent experiments, using four test articles: reference 3R4F cigarette smoke TPM, THP TPM, e-cigarette e-liquid and e-cigarette TPM.

Test articles were generated in a comparable manner for cigarette smoke, THP and e-cigarette TPMs, but at contrasting concentrations. Based on historical data and biological responses of reference 3R4F cigarette smoke, a standard stock concentration range was selected across the three treatment conditions, up to 20 mg/mL, resulting in a maximum concentration of 200 μg/mL (allowing for 1% v/v additions of the test article) which is comparable to previous studies (Combes et al., 2013; Crooks et al., 2013; Thorne et al., 2018a). THP and e-cigarette TPM stock concentrations were generated up to 50 mg/mL (giving final concentrations up to 500 μg/mL, allowing for 1% v/v additions of the test article). Finally, to further assess the potential of e-cigarettes to induce mutation, both the captured aerosol in the form of TPM and the e-liquid were assessed. This two-step process allowed the e-liquid to be assessed in its native form and upon aerosolisation, thus capturing the transformation of this liquid and any associated chemicals potentially generated through the aerosolisation and thermal degradation process. Unlike e-cigarettes with their associated e-liquid that is transformed upon aerosolisation, no such precursor exists for cigarette smoke and THPs, given that a tobacco precursor is combusted or heated to generate the aerosol. Therefore, TPM from cigarettes and THPs were solely assessed without a precursor. A further step could be to assess whole aerosol test articles in the MLA; unfortunately, due to limitations in technologies and methodologies, no such MLA-aerosol method currently exists.

3R4F TPM results clearly showed a positive induction in MF above the GEF under the three treatment conditions (3 h ± S9 and 24 h -S9). The observed responses were both concentration-related and reproducible across two independent experiments. Positive responses were observed at 3 h -S9 at concentrations exceeding 60 μg/mL TPM. At 3 h + S9, positive responses were observed at concentrations above 120 μg/mL TPM, and at 24 h -S9 with doses exceeding 20 μg/mL TPM. The MF values at each of these concentrations exceeded the GEF and,

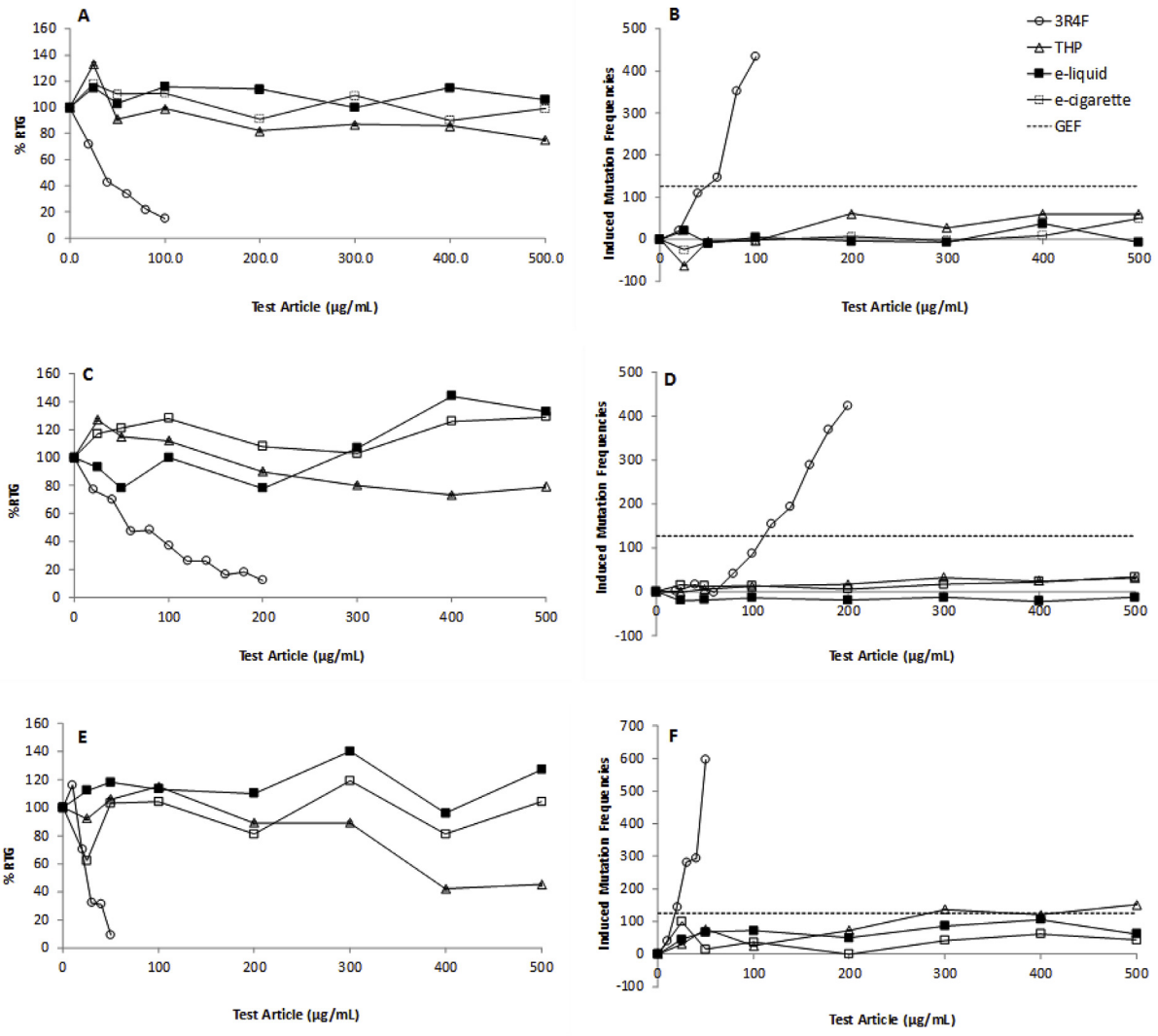

Fig. 1. % RTG and Induced mutant frequencies (IMF) under three treatment conditions, data expressed as a function of Test Article (TPM or e-liquid) concentration (µg/mL). (A) 3 h -S9% RTG; (B) 3 h -S9 IMF; (C) 3 h + S9% RTG; (D) 3 h + S9 IMF; (E) 24 h -S9% RTG; (F) 24 h -S9 IMF. Global evaluation factor (GEF) defined as 126 mutants per 10^6 viable cells. Example dataset from Experiment 1.

with one minor exception, were well within the 10–20% tolerances for RTG.

In contrast, THP TPM failed to produce a consistent and reproducible response. E-cigarette TPM and e-liquid responses were clearly negative even at the maximum concentration of 500 µg/mL. The RTG of the e-cigarette TPM and e-liquid did not fall below 60% and the RTG of the THP TPM did not fall below 40% even at the maximum concentration of 500 µg/mL, suggesting that in each case the exposure could be extended to higher concentrations. At the maximum concentration of 500 µg/mL (THP TPM), there was slight evidence of an increasing trend in induced MF, suggesting a possible imminent response. However, this response was only seen in Experiment 1 at 24 h -S9 but was not reproducible to the same magnitude (and fell below the GEF) in Experiment 2. The negative responses observed for the e-liquid and the aerosolised version of the same e-liquid confirmed that the aerosolisation of the e-liquid did not generate any spurious chemical species responsible for inducing mutation in this assay.

In addition to assessing cigarette smoke, THP and e-cigarettes using classical TPM and e-liquid preparation and dosing approaches (mass/mL), comparisons were also conducted on a nicotine equivalent basis. Using this approach, it is possible to compare vastly different products based on nicotine yield or content, which normalises for water, propylene glycol, vegetable glycerol, and other chemicals that may make up the majority of TPM mass. At the maximum concentrations of cigarette smoke under each treatment condition, the nicotine equivalent for the NGPs can be defined. This study calculated equivalent nicotine dosing across all products comparable to cigarette smoke and the equivalent TPM/e-liquid dose required to achieve this (Table 3). In terms of future research, this information will allow researchers to tailor their exposures to at least match for nicotine deliveries, which is especially important as each of the NGP categories have significantly different deliveries. Furthermore, the data demonstrate that nicotine is not the driving constituent for the observed genotoxic responses with cigarette smoke, as equivalent nicotine doses were demonstrated to have negative outcomes for the NGPs.

Previous studies have also investigated the mutagenic effect of NGPs, more specifically THPs. Schaller et al. (2016) concluded that a tobacco heating system was positive in the MLA assay at concentrations between 10 and 20% RTG. In this study using a comparable THP, we only generated ~40% RTG at the highest dose of 500 µg/mL, which was significantly higher in terms of TPM exposure and nicotine equivalents compared to that of cigarette smoke, with still no observed response. Interestingly, at ~40% RTG, a slight linear trend for increasing MF was observed in Experiment 1, suggesting as Schaller et al.

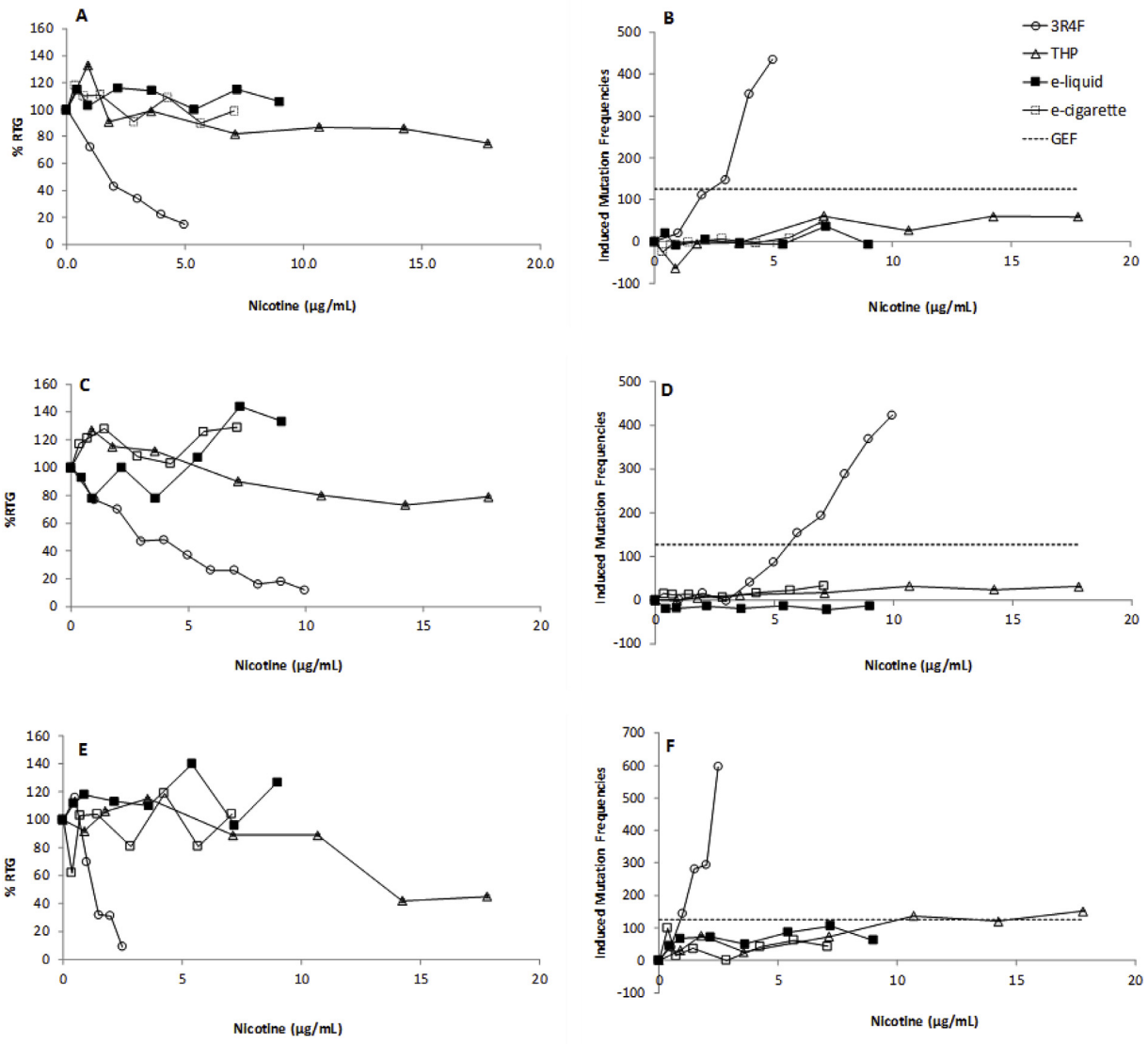

Fig. 2. % RTG and Induced mutant frequencies under three treatment conditions, data expressed as a function of nicotine concentration (µg/mL). (A) 3 h -S9% RTG; (B) 3 h -S9 IMF; (C) 3 h + S9% RTG; (D) 3 h + S9 IMF; (E) 24 h -S9% RTG; (F) 24 h -S9 IMF. Global evaluation factor (GEF) defined as, 126 mutants per 10^6 viable cells. Example dataset from Experiment 1.

Table 3
Nicotine equivalents.

Treatment condition	Test article	Product dose to achieve 3R4F equivalents (µg/mL)			
		3R4F (TPM)[a]	THP (TPM)[b]	e-cigarette (TPM)[b]	e-cigarette (e-liquid)[b]
3 h -S9	TPM	100	100	300	300
	Nicotine	4.98	3.56	4.25	5.4
3 h + S9	TPM	200	300	500	500
	Nicotine	9.95	10.68	7.08	9.00
24 h -S9	TPM	65	100	200	200
	Nicotine	3.23	3.56	3.83	3.60

[a] = Top dose of cigarette smoke TPM taken as the comparator dose.
[b] = The nicotine concentration of NGPs that closely matches the nicotine concentration of the 3R4F dose and the corresponding TPM/e-liquid dose to achieve equivalence.

(2016) demonstrated, that if THP exposures are pushed to higher levels, a response could be observed.

To our knowledge, there are no studies comparing the effects of e-liquid and the corresponding captured e-cigarette particulate fraction, and no studies comparing NGPs across categories as this study has done, using nicotine equivalents as a comparator. Although this study has assessed significantly higher concentrations than previously reported, in order to avoid reporting false-negatives, higher doses may still be required to fully assess the mutagenic potential of NGPs.

Table 4
Summary of findings.

Test Article	3R4F	THP[a]	E-Cigarette	E-Cigarette
Treatment condition	TPM	TPM	TPM	E-Liquid
3 h + S9	Positive	Negative	Negative	Negative
3 h -S9	Positive	Negative	Negative	Negative
24 h -S9	Positive	Negative	Negative	Negative

[a] Some observed increases, but not reproducible or considered biologically relevant.

6. Conclusions

This study has demonstrated a clear and reproducible

concentration-related cigarette smoke response (from 3R4F TPM) for the induction of MF in all three treatment conditions. Conversely, at TPM concentrations far exceeding those of cigarette smoke and at nicotine equivalent doses, THP and e-cigarette test matrices were all deemed to not consistently induce mutations in two independent experiments, with values falling below the GEF. This data set supports the use of the MLA assay for the assessment of NGPs (THP and e-cigarettes) and NGP test matrices (e-liquid and TPM).

This is Part One of a two-part series where the same test articles and TPM preparations were compared in both the MLA (detailed here) and the IVMN assay in a co-ordinated genotoxicity testing approach. In Part Two, Thorne et al. (2018b) compared the same test articles (using the same TPM and e-liquid preparations) in the *in vitro* micronucleus assay and demonstrated that even with an extended expression period of 24 + 24 h that neither THP (TPM) or e-cigarette (e-liquid or TPM) produced a positive response under any treatment condition. In contrast, cigarette smoke TPM produced a positive response under all three treatment conditions, which was further increased with the addition of an extended expression period. In summary, both the MLA and IVMN assays when tested with the same NGP test articles, produced negative findings, whilst cigarette smoke was consistently positive in both assays. This strengthens the understanding that both assays are equally valid and can be used interchangeably for the assessment of NGPs and that assessment of NGPs should be conducted using a weight of evidence approach with multiple assays to confirm real genotoxic potential.

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Abbreviations

DMSO	Dimethyl sulphoxide
E-cigarette	electronic cigarette
E-liquid	electronic cigarette liquid
ENDS	Electronic nicotine delivery system
GEF	Global evaluation factor
HCI –	health Canada intense smoking regimen
MF	mutant frequency
MLA	Mouse lymphoma assay
NGP	Next generation products
RTG	Relative total growth
THP	Tobacco heating products
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.2019.110584.

Authors contributions

The study was designed by Marianna Gaca, 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 Gaca, 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 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.

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