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In vitro mutagenicity of gas-vapour phase extracts from flavoured and unflavoured heated tobacco products

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ABSTRACT

The *in vitro* mutagenic and genotoxic potential of Heated Tobacco Products (HTPs) has already been studied with the particulate phase and reported previously. This study has been designed to complement the *in vitro* assessment of the HTP and to determine whether the inclusion of potential flavourings would alter the *in vitro* response by testing the other phase of the aerosol, the gas-vapour phase (GVP). Both flavoured and unflavoured Neostik GVP samples did not show any sign of mutagenic activity in the Ames test but induced a mutagenic response in the mouse lymphoma assay (MLA), however, these responses were significantly less than those of the reference cigarette, 3R4F. The results demonstrated that GVP emissions of this HTP did not induce either new qualitative or quantitative mutagenic hazards compared to 3R4F, as assessed by the Ames test (no new responsive strains) and MLA (a lower mutagenic response), respectively. A statistical comparative analysis of the responses showed that the addition of flavourings that may thermally decompose under the conditions of use did not add to the *in vitro* baseline responses of the unflavoured Neostik.

1. Introduction

Cigarette smoke is a complex mixture produced by the combustion and pyrolysis of tobacco and contains over 6000 identified chemical compounds [1]. Some of these compounds are well known to have toxicological properties that cause adverse health effects such as cardiovascular and respiratory disease and cancer [2–6]. It has been described that most of the known toxicants present in cigarette mainstream smoke results from the thermal decomposition at high temperatures of principally organic compounds present in tobacco [7,8].

In the early years of tobacco harm reduction, the focus was primarily on modifying the constituents of a conventional cigarette. For example, the tobacco could be modified by enzymatic treatment to reduce tobacco smoke toxicants precursors [9] or removing part of the tobacco portion and replacing it with a non-tobacco alternative, such as Tobacco Substitute Sheet containing high levels of glycerol and calcium carbonate [10]. Additionally, the aerosol delivered to a consumer could be modified by placing adsorbent material such as activated charcoal in the filter of a cigarette to adsorb gas-vapour constituents [11]. These technologies have demonstrated the potential to reduce toxicant emissions and these reductions were also reflected using *in vitro* mutagenicity and genotoxicity assays [12–14]. Studies have shown that reducing the temperature to which conventional tobacco is heated can reduce toxicant emissions [7,15]. This is typically achieved by modifying the way tobacco is heated. One of the early technologies to heat tobacco at a lower temperature used a carbon tip that allowed a heated stream of air to pass through the tobacco rod, resulting in a less complex aerosol with fewer toxicants. Indeed, this reduction in toxicants was also reflected *in vitro* by a significant reduction in the biological activity compared to commercial cigarettes [16]. Newer technologies use a device that heats but does not burn tobacco, termed Heated Tobacco Products (HTP).

British American Tobacco's device (glo™) consists of a rechargeable battery with heating elements controlled by a microprocessor in a handheld format and a Neostik (the HTP) that is composed of reconstituted Virginia blended tobacco and glycerol, with or without flavourings, forming the tobacco rod wrapped within a cigarette paper and a filter consisting of standard cellulose acetate with a hollow center and ventilation holes. After the Neostik is inserted into the heating chamber of the device, and following activation of the heating elements, it is heated to a maximum temperature of 250 °C. The user puffs on the filter end of the Neostik to inhale an aerosol that delivers nicotine and flavouring components, if present. This device and Neostik have been described in detail by Eaton et al. [17].

Heating of the Neostik to a considerably lower temperature than in

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conventional cigarettes results in the absence of combustion and pyrolysis and consequently a much less complex aerosol composition [15,18–21]. In comparison to the 3R4F reference cigarette, the toxicant levels in Neostik emissions were significantly reduced across all chemical classes analysed [22].

Even though the reduction of toxicants in the HTP category is a step forward in the potentially reduced risk product approach, this reduction in toxicants needs to be coupled with an acceptable sensorial experience to existing smokers when using the Neostiks. In order to offer traditional tobacco consumers a potentially less harmful product, flavourings are used in HTPs and these are typically the same as those used in conventional cigarettes. A series of flavouring ingredients has already been studied in cigarettes and they did not add to the toxicity of the base cigarette [23–26]. However, the chemical and toxicological properties of these flavourings used in the HTP environment and therefore heated to a lower temperature, is less well studied.

The risk assessment approach for ingredients used in Neostiks has been described by Eaton et al. [17]. Firstly, classified genotoxicants, non-threshold carcinogens, reproductive and developmental toxicants and respiratory sensitizers are excluded. A desk-based toxicological risk assessment is performed for ingredients that do not thermally decompose when heated to cigarette burning temperatures. Ingredients that may decompose when heated, or where no information is available, are assessed using analytical chemistry and *in vitro* studies.

In vitro testing can be used to complement analytical chemistry, confirming the reduction of toxicants results in a reduced biological effect [15,18,22]. Candidate flavouring ingredients for the HTP category that may decompose under the HTP conditions of use, or where no information was available, have been listed and their highest foreseeable level in use described (Table 1). All these flavouring ingredients at their highest foreseeable level of use were combined into the tobacco rod of a single Neostik. A reference Neostik that did not contain any flavouring but the same tobacco base blend, cigarette paper and filter was also manufactured as the control.

The Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) *In vitro* Toxicology Task Force recommended [27] to include the following tests for conducting *in vitro* toxicology testing of tobacco smoke.

Table 1
Flavourings added to the flavoured Neostik and the maximum inclusion level.

Name	CAS No.	% Inclusion (w/w relative to tobacco)
Cinnamic acid	621-82-9	0.001–0.01%
Guaiacol	90-05-1	
Hexanoic acid	142-62-1	
Lime oil, terpeneless	68916-84-7	
Methyl butyraldehyde (3-)	590-86-3	
Methyl cyclopentenolone	80-71-7	
Phenyl-2-propen-1-ol (3-)	104-54-1	
Anethole (trans-)	4180-23-8	0.01–0.1%
Cinnamaldehyde	104-55-2	
Cinnamyl cinnamate	122-69-0	
Citral	5392-40-5	
Dodecalactone (delta-)	713-95-1	
Ethyl maltol	4940-11-8	
Eugenol	97-53-0	
Limonene (d-)	5989-27-5	
Methyl benzyl acetate (alpha-)	93-92-5	
Pepper oil, black	8006-82-4	
Peppermint oil	8006-90-4	
Piperonal	120-57-0	
Spearmint oil	84696-51-5	
Vanillin	121-33-5	
Undecalactone (gamma-)	104-67-6	
Mandarin oil	8008-31-9	0.1–1.0 %
Orange oil	8028-48-6	
Tangerine oil	8016-85-1	

- The Ames test, a bacterial mutagenicity test that detects point mutations in DNA [28].
- The *in vitro* micronucleus test, a mammalian cell mutation assay that measures chromosomal changes in the form of aneugens (changes to chromosome number) or clastogens (changes to chromosome structure) [29].
- The *in vitro* mouse lymphoma assay (MLA), another mammalian cell mutation assay that also detects chromosomal changes and measures point mutations in mammalian cells [30].
- The Neutral Red uptake assay, a cellular cytotoxicity assay based on the ability of viable cells to incorporate a supravital dye [31].

These recommendations are broadly in accordance with the strategies describing the use of core *in vitro* mutagenicity and genotoxicity assays for the detection of mutagenicity and genotoxicity developed by the International Conference on Harmonisation [32] and Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment [33]. According to ICH recommendations, when integrated in an *in vitro* testing strategy with other genotoxicity assays, the MLA and the *in vitro* micronucleus are mutually acceptable and interchangeable as the mammalian cell assay for the detection of chromosomal damage.

The *in vitro* mutagenicity, genotoxicity and cytotoxicity testing of tobacco products has historically focussed on testing the particulate phase (TPM). The testing of TPM allow products to be compared and represents a reliable, consistent and reproducible test matrix even with the large variety of collection methods available [34]. However, cigarette smoke consists of two distinct phases. These are TPM and the gas-vapour phase (GVP), consisting of volatile and semi-volatile compounds, and is comprised of 400–500 chemicals [35]. Some of these compounds are well known toxicants [2,36].

A standardized method has been developed to capture water soluble GVP constituents (Health Canada Official Method T-502 Appendix I). This method consists of puffing a cigarette on a smoking machine using standard methods. The particulate phase is captured on the Cambridge Filter Pad (CFP), and studies have shown that the CFP is 99.9% effective at capturing the TPM [37]. The soluble GVP constituents that pass through the CFP are collected by bubbling into phosphate-buffered saline (PBS). The Health Canada method of GVP collection is usually used for cytotoxicity testing, however, there is no reason why it cannot be applied to other *in vitro* assays. Indeed, this preparation of GVP, or slightly modified alternatives thereof, has been used to test heat not burn tobacco products and cigarettes in *in vitro* toxicology assays, as described in other publications [38,39].

Large amounts of data have been generated on testing TPM from cigarette mainstream smoke and the assessment of TPM from HTP mainstream aerosol is an emerging field. However, there is little published information on the *in vitro* GVP fraction mutagenic potential. This study was designed to test the hypothesis that the addition of flavouring ingredients in a Neostik does not add to the HTP GVP baseline *in vitro* responses. This publication presents the results of a bacterial reverse mutation assay (Ames test) and a mammalian *in vitro* mutagenicity assay (mouse lymphoma assay) performed with flavoured and unflavoured Neostiks and the reference cigarette, 3R4F, GVP to complement the TPM mutagenic potential previously described in Crooks et al. [18].

2. Materials and methods

2.1. Cigarettes and Neostiks

3R4F cigarettes, an American-blend filtered reference cigarette with a length of 84 mm and circumference of 24.8 mm, were purchased from the University of Kentucky Research and Development Center (Lexington, KY, USA).

Both flavoured and unflavoured Neostiks used in this study were

manufactured by British American Tobacco, Southampton, UK. All Neostiks were of 15.7 mm circumference, had 42.0 mm tobacco rod length and contained a reconstituted Virginia tobacco blend with glycerol (14.8%). HTP candidate flavours were included in the tobacco rod of the flavoured Neostik (Table 1) at the highest foreseeable use level.

2.2. Generation of gas-vapour phase

Batches of GVP samples were prepared aseptically as described in Health Canada Official Method T-502-Appendix I [52], using sterile solutions and no or low UV lighting in the rooms where the sample generation and sample analysis were conducted. 3R4F cigarettes, Neostiks and 44 mm Cambridge filter pads (Whatman, Maidstone, UK) were conditioned according to ISO 3402 [40].

All products were puffed on Borgwaldt RM200 s smoking machines (Borgwaldt-KC, Hamburg, Germany). 3R4F cigarettes were smoked under the Health Canada intense regime [41], namely 55 mL puff volume, 30 s puff interval with 100% vent blocking. In Neostiks, the filters had ventilation holes that are designed to reduce the vapour temperature [17]. When in use, the proximity of the ventilation holes to the device means that it is not possible to block the ventilation holes [42] and therefore, the Health Canada intense regime was adapted, so that the Neostik ventilation holes were 100% open during puffing. To prepare the GVP from Neostiks, the Neostiks were inserted into the HTP device, switched on and puffed on a Borgwaldt RM200 s smoking machine, as per the procedure below.

The Borgwaldt RM200 s smoking machine was set up such that each puff passed through a 44 mm Cambridge Filter Pad (CFP) to remove particulates, and the resulting gas-vapour phase (GVP) was bubbled through 15 mL of sterile ice-cold calcium and magnesium-free PBS in an appropriate impinger held on ice. Cambridge filter pads were changed before breakthrough occurred (*i.e.* to a maximum pad loading of approximately 150 mg TPM, as per the recommendation of ISO, 4387: 2000 [43]). Each pad was weighed, pre and post puffing/smoking such that a total TPM weight of the smoking run could be determined. This allowed the final GVP sample concentration to be calculated as mg TPM equivalent per mL PBS (mg TPM eq./mL), and by addition of the required further volume of ice-cold PBS, the concentration of each GVP sample was adjusted to 50 mg TPM eq./mL. This stock concentration was selected to reach the maximum final concentrations recommended by OECD guidelines of 5000 μg/plate for the Ames assay and 5000 μg/mL in the mouse lymphoma assay for chemical substances of Unknown or Variable Composition (UVCBs).

In order to avoid potential cross contamination, devices used for the flavoured and unflavoured Neostiks were puffed on separate RM200 s smoking machines than 3R4F cigarettes. All HTP devices were checked to ensure they were working according to specification before generation of GVP samples.

All GVP samples were stored in sealed brown bottles with minimum headspace and refrigerated (2–8 °C) immediately after generation. Each GVP sample was tested within 3 h of preparation.

For the determination of nicotine, water and glycerol, three pads were randomly sampled from each day of GVP generation, per product. Pads were placed in isopropyl alcohol (Sigma-Aldrich, Poole, UK) and nicotine, water and glycerol were measured by gas chromatography with a thermal conductivity detector and a flame ionisation detector.

2.3. In vitro toxicology testing

All *in vitro* assays were conducted according to Good Laboratory Practice. Appropriate vehicle and positive controls were used concurrently for each assay and treatment condition. All chemicals were purchased from Sigma-Aldrich.

2.3.1. Ames test

The bacterial reverse mutation assay was performed in accordance with OECD 471 Test Guideline recommendations [54]. The GVP of 3R4F and both the unflavoured and flavoured Neostiks were tested in five histidine-requiring strains TA98, TA100, TA1535, TA1537 and TA102 of *Salmonella typhimurium*. Strains TA98, TA1535 and TA1537 were originally obtained from the United Kingdom National Collection of Type Cultures (UK NCTC). Strains TA100 and TA102 were derived from cultures originally obtained from Covance Laboratories Inc., USA.

Three independent experiments were performed both in the absence and the presence of metabolic activation (S9; Aroclor 1254-induced rat liver post-mitochondrial supernatant mix). One of these experiments consisted of a direct plate incorporation of the GVP samples and the two other experiments consisted of a 60-minute pre-incubation before the addition of the molten agar, in order to maximise the exposure of GVP to the assay system. For all assays, bacteria were cultured at 37 ± 1 °C for 10 h in nutrient broth, supplemented with ampicillin (TA98, TA100) or ampicillin and tetracycline (TA102) to provide a bacterial range of approximately 10^8 to 10^9 cells/mL before treatment.

In the absence of S9, each tester strain was tested in triplicate plates. In the presence of S9, triplicate plates were used for strains TA1535 and TA102, quadruplicate plates for TA100, quintuplicate plates for TA98 and ten plates per concentration were treated for strain TA1537 as recommended by Scott et al. [44]. At least 6 concentrations were tested in each treatment condition and experiment.

The positive controls used in experiments in the absence of S9 were 2-nitrofluorene (5 μg/plate) for TA98, sodium azide (2 μg/plate) for TA100 and TA1535, 9-aminoacridine (50 μg/plate) for TA1537 and Mitomycin C (0.2 μg/plate) for TA102. In the presence of S9, the positive controls were benzo[*a*]pyrene (10 μg/plate) for TA98 and 2-aminoanthracene for TA100, TA1535, TA1537 (5 μg/plate) and TA102 (20 μg/plate).

GVP samples were tested up to 5000 μg TPM eq./plate and treatment was achieved by adding 0.1 mL of bacterial culture, 0.1 mL of test article/vehicle control or 0.05 mL of positive control and 0.5 mL of 10% S9 mix or buffer solution as appropriate, to 2 mL of supplemented molten agar at 45 ± 1 °C. This preparation was then poured on to Vogel-Bonner E agar plates.

When set, the plates were inverted and incubated at 37 ± 1 °C protected from light for 3 days. Following incubation, the background lawn of the plates were examined for signs of toxicity and revertant colonies were counted electronically using a Sorcerer Colony Counter (Perceptive Instruments) or manually where required. Individual plate counts were recorded, and the mean and standard deviation of the plate counts were determined for each concentration.

The assay was considered to be valid if the vehicle control counts fell within the laboratory's historical control range and positive controls induced increases in revertant numbers of ≥1.5 fold (in strain TA102), ≥ 2-fold (in strains TA98 and TA100) or ≥ 3-fold (in strains TA1535 and TA1537) the concurrent vehicle control.

GVP samples were considered mutagenic if a reproducible increase in revertant numbers gave a statistically significant response when assessed using Dunnett's test, and this increase in the number of revertant was considered to be concentration related.

2.3.2. Mouse lymphoma assay

The MLA was performed in accordance with OECD Test Guideline 490 [53], using L5178Y $tk^{+/-}$ cells (originated from Dr Donald Clive, Burroughs Wellcome Co.) maintained in RPMI10 medium: Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 10% v/v heat inactivated horse serum, L-glutamine, HEPES, penicillin (100 units/mL), streptomycin (100 μg/mL), amphotericin B (2.5 μg/mL), pluronic acid (0.5 mg/mL) and sodium pyruvate (0.2 mg/mL).

The GVP from 3R4F and both the unflavoured and flavoured Neostiks were tested in two independent experiments up to 5000 μg TPM eq./mL as recommended by OECD Test Guideline 490 for UVCB substances. Each experiment was composed of two 3 h exposure treatments, one in the presence and one in the absence of S9 (Aroclor 1254-

induced rat liver post-mitochondrial supernatant mix), and one 24 h exposure treatment in the absence of S9. At least 9 concentrations were tested per GVP sample, with four replicate cultures per concentration as recommended by Scott et al. [44]. Duplicate cultures were used for the positive controls. Appropriate vehicle and positive controls were used in parallel for each treatment condition. The positive controls used were 15 and 20 μg/mL methyl methane sulphonate in the 3 h exposure in the absence of S9, 5 and 7.5 μg/mL methyl methane sulphonate in the 24 h exposure in the absence of S9, and 2 and 3 μg/mL benzo[*a*]pyrene in the 3 h exposure in the presence of S9.

Treatments were performed by adding 2 mL of vehicle or GVP sample or 0.2 mL of positive control solution (completed with 1.8 mL calcium and magnesium-free PBS) into the appropriate volume of culture medium to achieve a final treatment volume of 20 mL, containing at least 10^7 cells or 4×10^6 cells for the 3 and 24 h treatments, respectively. The 3 -h treatment culture medium was reduced to 5% serum (RPMI5) and complemented by S9 mix or 150 mM potassium chloride (KCl) as appropriate. Cell cultures were then incubated at 37 ± 1 °C in a humidified incubator gassed with 5 ± 1% v/v CO_2 in air for the designated exposure period.

Following the appropriate incubation period, cells were washed twice, and cell densities adjusted to 2×10^5 cells/mL in RPMI10 and transferred to tissue culture flasks for growth for a further 2-day incubation; the expression period.

At the end of the expression period, cells were adjusted to 1×10^4 cells/mL and approximately 8 cells/mL in RPMI20 (RPMI complemented with 20% serum) before being plated for TFT resistance and viability, respectively. Two 96-well microtitre plates were used for viability assessment (192 wells averaging 1.6 cells/well) and four 96-well microtitre plates were used to assess TFT resistance (384 wells at 2×10^3 cells/well), by the addition of 3 μg/mL TFT. All plates were incubated at 37 ± 1 °C in a humidified incubator gassed with 5 ± 1% v/v CO_2 in air until scoreable. Wells containing viable clones were identified by eye using background illumination and counted. Cytotoxicity was determined using the Relative Total Growth (RTG). Concentrations inducing a reduction of viability below 10% RTG were excluded from the mutation analysis as recommended by the OECD Test Guideline.

The assay was considered valid if the concurrent vehicle control's mutation frequency (MF) was within the historic vehicle control ranges of the laboratory and positive control showed an absolute increase in mean total MF of at least 300×10^{-6}. Instances that did not fulfil these criteria were evaluated on a case-by-case basis.

Data were evaluated as per the recommendations in Moore et al. [45]. A GVP was considered mutagenic if the mutation frequency of any test concentration exceeded the sum of the vehicle control mutant frequency plus the Global Evaluation Factor (GEF); the linear dose-response was statistically significant, and the observed response was reproducible under the same treatment conditions.

Graphs were prepared in GraphPad Prism 7.01 using a four-parameter variable slope fit and the concentrations inducing a positive response in the MLA (vehicle control mutation frequency plus the GEF) were calculated based on the fitted curve.

2.3.3. Comparative analysis

Statistical comparative analyses of flavoured and unflavoured GVP mutagenic activity in the mouse lymphoma assay were performed as per Scott et al. [44] recommendations for each independent experiment, providing that for each comparison both GVP test items were mutagenic and provided a concentration related response.

3. Results

3.1. GVP generation

The GVP generation from the flavoured and unflavoured Neostiks and 3R4F for use in the *in vitro* assays was performed on Borgwaldt RM200 s machines and puffed to the puffing parameters of the Health Canada Intense regime (modified for HTPs with ventilation holes not blocked). The mean values and standard deviations for TPM, nicotine, water and glycerol per stick are shown in Table 2.

Table 2
Mean values expressed as mg/stick (± Standard Deviation) for TPM, nicotine, glycerol and water.

	3R4F		Flavoured Neostik		Unflavoured Neostik	
	Mean	± SD	Mean	± SD	Mean	± SD
TPM (mg/stick)	36.89	1.51	16.24	1.71	16.05	2.01
Nicotine (mg/stick)	1.90	0.10	0.41	0.07	0.40	0.07
Glycerol (mg/stick)	2.36	0.22	3.46	0.62	3.44	0.68
Water (mg/stick)	11.48	0.92	9.11	1.27	9.01	1.57

3.2. Ames test

The vehicle and positive controls in each experiment control were within the historical range of the revertant numbers for the laboratory and the positive controls induced increases in revertant numbers of ≥1.5 fold (in strain TA102), ≥ 2-fold (in strains TA98 and TA100) or ≥ 3-fold (in strains TA1535 and TA1537) the concurrent vehicle control therefore the assay was considered valid.

Evidence of toxicity (thinning of the background bacterial lawn and/or reduction in the number of revertants) was observed with GVP from 3R4F in experiments using the pre-incubation methodology. This occurred at the highest tested concentrations in strain TA98 and TA1537 in the absence of S9. No other evidence of toxicity was observed in any other strain, treatment condition or experiment with any of the other GVP samples.

3R4F GVP samples induced small, dose related but statistically significant ($p \leq 0.01$) and reproducible increases in revertant numbers in TA100 when tested in both the absence and presence of S9 (Fig. 1). Some other statistically significant increases ($p \leq 0.01$) in revertant numbers were observed in some other tester strains when treated with GVP samples of 3R4F, but none of these were reproducible, and in most cases were not concentration-related.

No reproducible concentration-related increases in revertant numbers were observed in any tester strain, under these treatment conditions, for flavoured and unflavoured Neostik GVPs up to 5000 μg TPM eq./plate in three independent experiments with 5 tester strains of *S. typhimurium* with or without S9 (Fig. 1), under the experimental conditions described.

3.3. MLA

The GVP samples from the flavoured and unflavoured HTP Neostiks and 3R4F reference cigarette were tested in the MLA with L5178Y $tk^{+/-}$ cells for 3 h and for 24 h in the absence of S9 and for 3 h in the presence of S9 in two independent experiments. The vehicle control counts were within the historical control ranges of the laboratory. The only exception to this was observed in the 24 -h treatment in the absence of S9 in experiment 1, where the mean vehicle control MF was slightly above the historical vehicle control MF range. However, the positive controls induced statistically significant increases in the mutation frequency of at least 300×10^{-6} demonstrating the sensitivity of the assay.

All GVP samples produced concentration dependent cytotoxicity as shown by decreases in Relative Total Growth (RTG), reaching the maximum cytotoxicity recommended by the OECD Guideline (20–10% RTG) in each treatment condition compared to the vehicle control. The

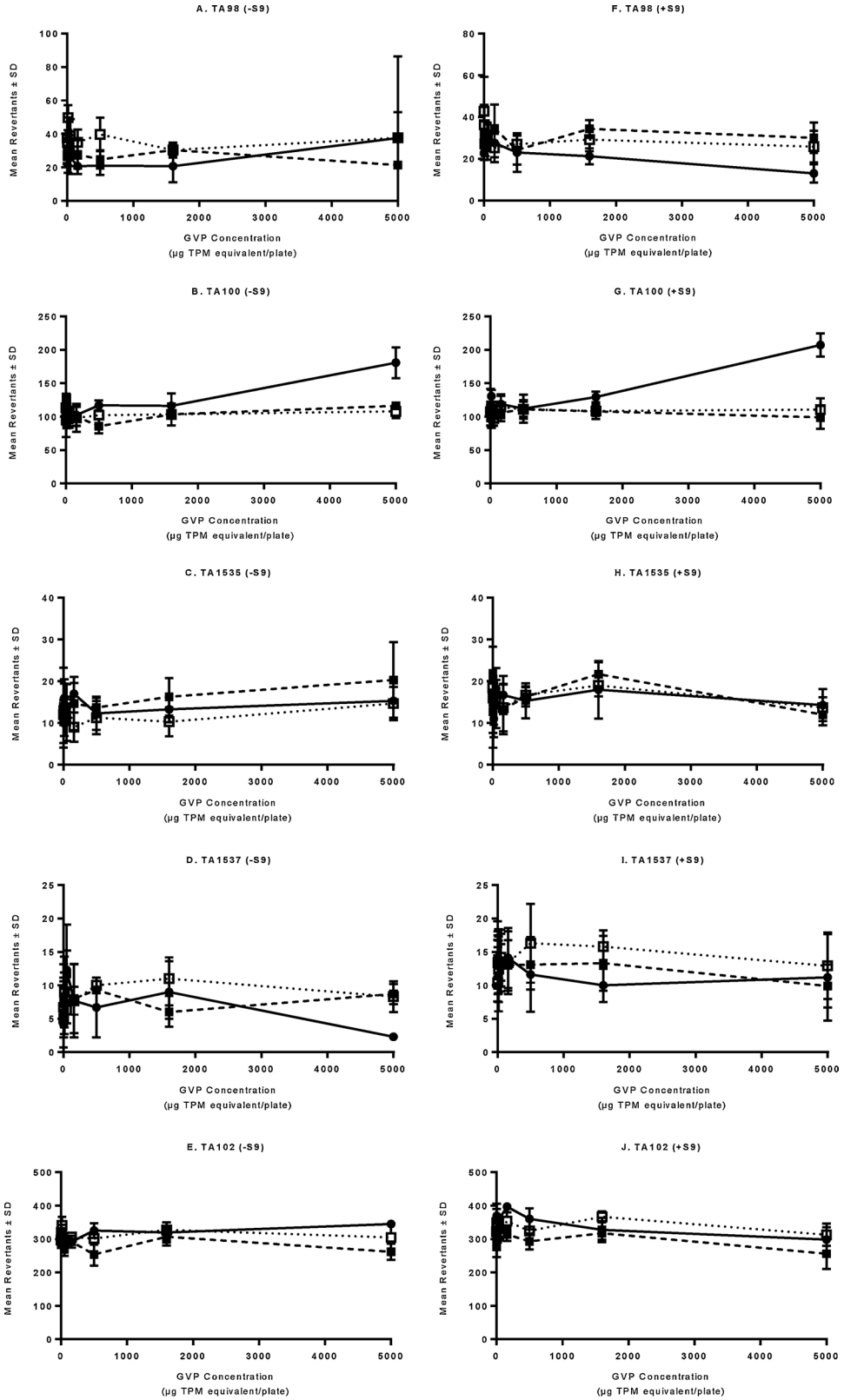

Fig. 1. Responses to GVPs from 3R4F reference cigarettes (●) and flavoured (■) and unflavoured (□) THP Neostiks in the Ames test, with tester strains TA98 -S9 (**A**), TA100 -S9 (**B**), TA1535 -S9 (**C**), TA1537 -S9 (**D**), TA102 -S9 (**E**), TA98 + S9 (**F**), TA100 -S9 (**G**), TA1535 + S9 (**H**), TA1537 + S9 (**I**) and TA102 + S9 (J) from one experiment with the pre-incubation method, as a representative example. The error bars represent the Standard Deviation (SD) of the replicate plates per concentration. Error bars not visible are contained within the data point. Data from one experiment are presented as representative data of the overall assay results.

Table 3
Highest concentrations (expressed as µg TPM eq./mL) inducing a decrease of % Relative Total Growth between 20 and 10% in the MLA for each tested exposure condition.

	3 hour (-S9)		3 hour (+S9)		24 hour (-S9)	
	1st Exp.	2nd Exp.	1st Exp.	2nd Exp.	1st Exp.	2nd Exp.
3R4F (µg TPM eq./mL)	130	150	250	275	120	100
Flavoured Neostik (µg TPM eq./mL)	1300	2000	2250	2000	1000	1300
Unflavoured Neostik (µg TPM eq./mL)	1500	1500	1600	2000	1000	1100

highest tested concentrations analysed for mutagenic activity were the concentrations inducing this maximum acceptable cytotoxicity (described in Table 3 and Fig. 2). 3R4F GVP samples induced extreme cytotoxicity at concentrations approximately 10-times lower than Neostik GVP samples. The addition of flavourings to the Neostik did not change the cytotoxic response induced by the unflavoured Neostik GVP samples.

3R4F GVP samples induced concentration-related increases in mutation frequency that exceeded the Global Evaluation Factor (GEF) plus the concurrent vehicle control (Fig. 3 and Table 4) and there was a highly significant linear trend ($p \leq 0.05$), however there were two exceptions. In experiment 1, 24 h -S9, no increases in mutation frequency were observed, and in the 3 h -S9 treatment, biologically relevant increases in mutation frequency were observed, however no concentrations exceeded the GEF plus vehicle the control.

The GVP generated from flavoured and unflavoured Neostiks induced concentration-related increases in mutation frequency that exceeded the GEF plus the concurrent vehicle control, in each experiment and treatment condition (Fig. 3 and Table 4) and there was a highly significant linear trend ($p \leq 0.05$), with the exception of the 24 -h treatment in experiment 1 with the flavoured HTP GVP which did not show a statistically significant linear trend.

The comparative statistical analysis between the flavoured and unflavoured Neostik GVP mutagenic responses following the 24 -h treatment in the absence of S9 indicated that the slope or magnitude of response for GVP from unflavoured Neostik was statistically significantly greater ($p \leq 0.05$) than for flavoured Neostik GVP. Statistical comparisons between flavoured and unflavoured Neostik GVP mutagenic responses following the 3 -h treatments in the absence and presence of S9 demonstrated that they were not statistically significantly different to each other. Furthermore, there is a clear difference between the response from 3R4F and both Neostik responses, with the Neostik responses showing a substantial reduction in *in vitro* mutagenic potency compared to that of 3R4F.

4. Discussion

The objective of this study was to test the hypothesis that, flavour ingredients do not add to the *in vitro* mutagenicity of the baseline HTP GVP and to complement the TPM mutagenic potential previously assessed [18]. The GVP generated from the flavoured and unflavoured Neostiks were tested in a suite of *in vitro* mutagenicity assays. They were compared to each other and with the response to GVP from a

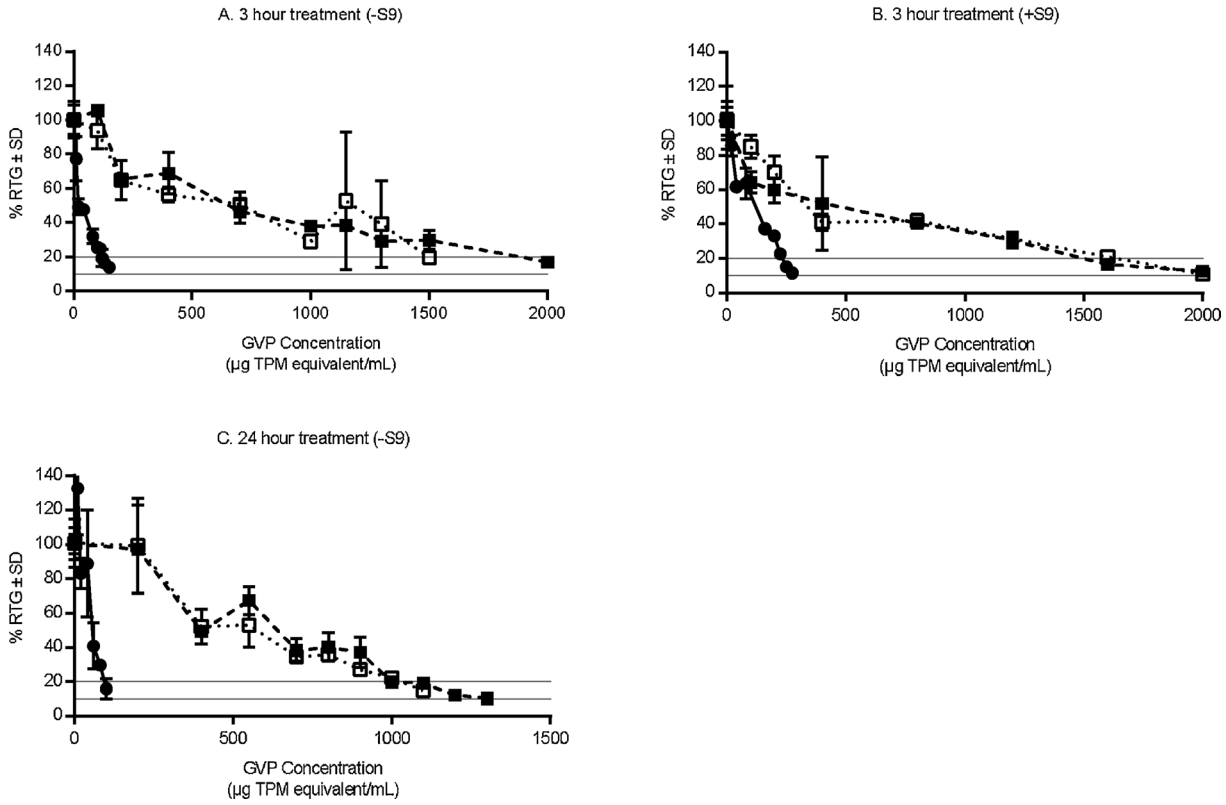

Fig. 2. Percent Relative Total Growth (RTG) of GVPs from 3R4F reference cigarettes (●), flavoured (■) and unflavoured (□) THP Neostiks in the MLA with L5178Y $tk^{+/-}$ cells. **A.** %RTG following a 3 -h exposure without metabolic activation; **B.** %RTG following a 3 -h exposure with metabolic activation; **C.** %RTG following a 24 -h exposure without metabolic activation. The error bars represent the Standard Deviation (SD) of 4 replicate cultures per concentration. Error bars that are not visible are contained within the data point. Black plain lines represent the limits of acceptable cytotoxicity (between 20 and 10% RTG) recommended by OECD Guideline 490. Data from one experiment is presented as representative of the overall assay results.

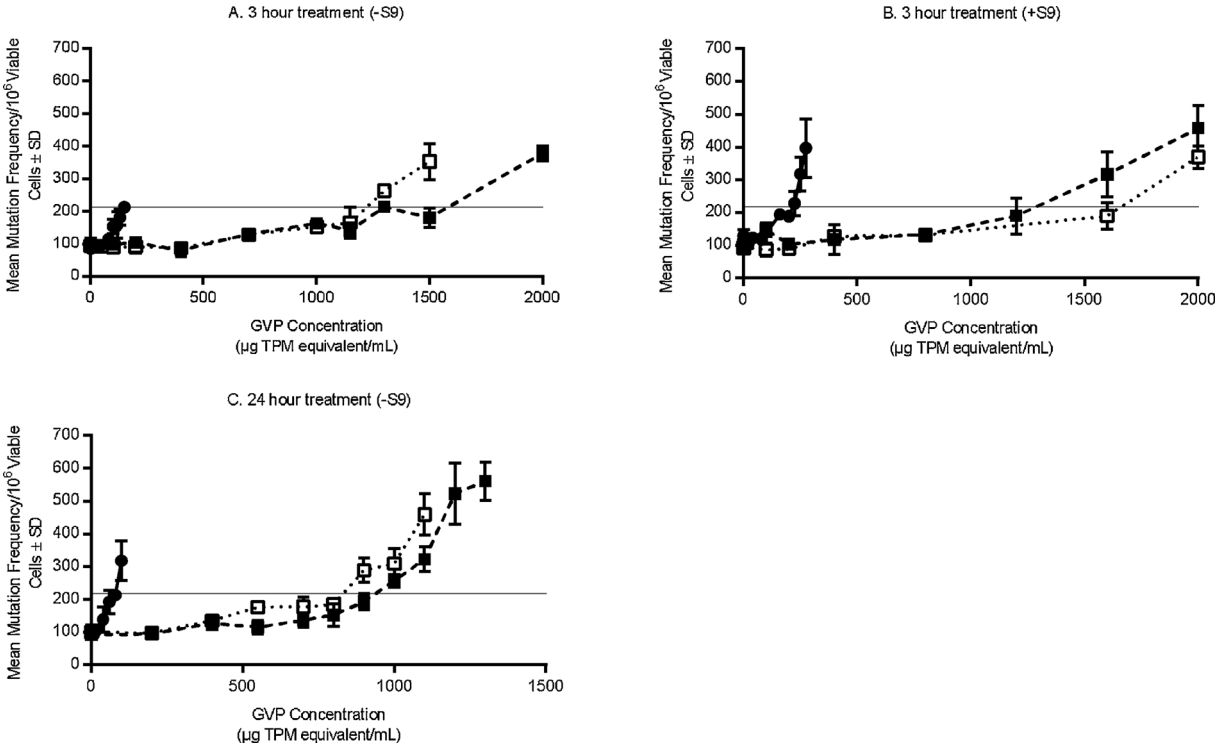

Fig. 3. Mutagenic responses (per 10^6 cells) (MF) to GVPs from 3R4F reference cigarettes (●), flavoured (■) and unflavoured (□) THP Neostiks in the MLA with L5178Y tk$^{+/-}$ cells. **A.** MF following a 3 -h exposure without metabolic activation; **B.** MF following a 3 -h exposure with metabolic activation; **C.** MF following a 24 -h exposure without metabolic activation. The error bars represent the Standard Deviation (SD) of 4 replicate cultures per concentration. Error bars that are not visible are contained within the data point. The black plain line represents the positive threshold (vehicle control MF + Global Evaluation Factor). Data from one experiment is presented as representative of the overall assay results.

Table 4
Calculated concentration (expressed µg TPM eq./mL) inducing a positive response (Positive response threshold = vehicle control MF + GEF) in the MLA for each tested exposure condition.

	3 hour (-S9)		3 hour (+S9)		24 hour (-S9)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
3R4F (µg TPM eq./mL)	*Not reached*	150	182	229	*Not reached*	78
Flavoured Neostik (µg TPM eq./mL)	1070	1525	1154	1338	950	951
Unflavoured Neostik (µg TPM eq./mL)	997	1237	1023	1684	914	842

reference cigarette, 3R4F following generation under the Health Canada intense puffing parameters (modified to have unblocked ventilation holes in the HTPs).

4.1. GVP in vitro toxicology

4.1.1. Ames test

3R4F GVP provided increases in revertant numbers in the histidine-requiring strain TA100 of *Salmonella typhimurium*, when tested in both the absence and presence of S9 under the conditions employed for this study. These reproducible statistically significant increases in the number of revertants were considered as evidence of weak mutagenic activity, that did not require metabolic activation. The sporadic increases observed in other tester strains in the Ames test were either not reproducible, or not dose related and therefore were not considered as evidence of mutagenic activity. Reference cigarette GVP has been reported to induce mutagenic responses in tester strains TA100 (Stabber et al., 2017; [46]), TA98 and TA100 although the exposure methods were variable, such as direct exposure using a CULTEX-B exposure module [47,48] or bubbling GVP directly into the bacterial suspension [49].

Neither flavoured nor unflavoured HTP GVP samples induced any biologically relevant increase in the number of revertants when tested with the histidine-requiring strains TA98, TA100, TA1535, TA1537 and TA102 of *Salmonella typhimurium,* at concentrations up to 5000 µg TPM eq./plate (the maximum concentration recommended by OECD 471 for UVCBs), both in the absence and presence of S9, and therefore did not show any mutagenic activity in the Ames test.

The absence of mutagenicity in the Ames test could be explained by the reduction of constituents in the test matrix. According to Stabbert et al. [50], of the 15 constituents representing approximately 87% of the chemicals trapped into the GVP, 6 of these compounds (acrolein, crotonaldehyde, methyl vinyl ketone, formaldehyde, 2,3-butanedione and methacrolein) tested positive for bacterial mutagenic activity with strain TA100. Acrolein, crotonaldehyde and formaldehyde accounted for approximately 66.6% of the mutagenicity of 2R4F GVP (a reference cigarette with no significant difference to 3R4F in terms of *in vitro* responses, according to Roemer et al. [25,26]). The chemical analysis of the whole aerosol of both the flavoured and unflavoured Neostik [18] showed that acrolein and crotonaldehyde were below the Limit of Quantification (LOQ) of 0.77 and 0.16 µg/Neostik, for these analytes, respectively. These compounds are present at concentrations equal to at least 196 times the LOQ in the 3R4F analysis (151.07 and 55.20 µg/cig for acrolein and crotonaldehyde, respectively). Formaldehyde is present at concentrations above the LOQ in Neostiks mainstream aerosol but is 37-fold higher in 3R4F mainstream smoke than in flavoured and unflavoured HTP Neostik aerosols (1.52 ± 0.15 and 1.79 ± 0.28 µg/cig,

respectively). Therefore, the consequent reduction of these 3 aldehydes, as well as other constituents in the aerosol relative to 3R4F, could be contributing to the absence of mutagenic response in the TA100 when tested with the HTP GVP samples (methyl vinyl ketone, 2,3-butanedione and methacrolein were not part of the whole aerosol chemical analysis described by Crooks et al. [18]).

4.1.2. MLA

The 3R4F cigarette GVP provided biologically, statistically significant and reproducible responses in all the treatment conditions with or without metabolic activation in the MLA. Data on the mutagenic activity of 3R4F GVP samples in the MLA are limited in the scientific literature. Wittke and Trelles-Sticken [51] and Schaller et al. [38] have described mutagenic activity induced by 3R4F GVP samples in the MLA showing that the MLA was sensitive enough to detect mutagenic activity of the GVP sample of cigarette mainstream smoke. The responses observed both with 3R4F, flavoured and unflavoured Neostik GVPs are consistent with the results described by Schaller et al. [38]. Indeed, both flavoured and unflavoured Neostik GVP samples also induced mutagenic responses in the mouse lymphoma assay under the test conditions described here. The concentrations required to induce a positive response (using the GEF plus vehicle control as a benchmark) both with flavoured and unflavoured Neostiks GVPs were approximately 5 to 12-times higher than 3R4F GVP concentrations (Table 4), demonstrating the lower *in vitro* mutagenic potency of the Neostiks relative to 3R4F GVP.

A statistical comparative analysis of the responses showed that the addition of flavourings that may thermally decompose in the Neostik, did not add to the *in vitro* baseline responses of the unflavoured Neostik.

5. Conclusion

The *in vitro* mutagenicity potential of this HTP has been further characterised with the testing of the GVP samples of both unflavoured and flavoured Neostiks. The data presented here showed that GVP samples are amenable to testing in Ames and MLA as signs of toxicity and mutagenic activity were observed in the Ames test following treatment with 3R4F GVP and the MLA when treated with 3R4F and HTP GVP.

In vitro responses to the HTP GVPs were absent in the Ames test and 5 to 12-times lower in the mouse lymphoma assay, compared to 3R4F GVP, demonstrating reduced mutagenic activity of the HTP GVP. The HTPs GVPs, compared to 3R4F GVP, did not induce either new qualitative or quantitative mutagenic hazards, as assessed by the Ames (no new responsive strain and absence of response in strains sensitive to combustible products) and MLA (lower mutagenic response), respectively.

Overall, the reduction of measured toxicants in Neostik emissions are consistent with a reduction of the mutagenic activity observed in the MLA compared to 3R4F cigarettes (GVP samples), and the absence of response to the HTP GVP samples in the Ames test. The HTP GVP response did not show any statistically significant different response in any assay between flavoured and unflavoured Neostiks with the exception of the 24 -h treatment in the absence of S9 in the MLA indicating that the magnitude of response for GVP from unflavoured Neostik was greater than for flavoured Neostik GVP. Therefore, the addition of flavourings to the Neostik, that thermally decomposed at cigarette burning temperatures, did not add consistently to the *in vitro* mutagenicity/genotoxicity baseline response to the HTP GVP, under the experimental conditions described.

Declaration of Competing Interest

None.

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