The effect of flavourings on the *in vitro* mutagenic and genotoxic potential of total particulate matter and gas vapour phase generated from a tobacco heating product

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Introduction

- A development prototype Tobacco Heating Product (THP) has been developed that heats a tobacco rod (consumable) up to 240°C, releasing water, nicotine, glycerol and if present, flavourings.
- The lower heating temperature and absence of combustion results in an aerosol that is less complex compared to a traditional combustible cigarette[1, 2].
- Flavourings have been added to the development prototype consumable, some of which may thermally decompose upon heating.
- However, to assess the genotoxic hazards that such products may present and to determine whether any flavouring ingredients that may thermally decompose effect the baseline toxicity profile, the particulate (TPM) and gas vapour phase (GVP) of development prototype flavoured and unflavoured consumables were tested in the Ames test and mouse lymphoma assay.
- The reference combustible tobacco product, 3R4F, was also included for comparison.

Materials and Methods

Consumables and Cigarettes

- The flavoured consumable contained flavourings commonly applied to tobacco products, that are expected to thermally decompose at cigarette burning temperatures[3]. The unflavoured consumable was composed of the same components but did not include any flavourings.
- 3R4F cigarettes were used as a reference combustible product.

TPM and GVP Generation

- Products were puffed to the HCI regime and TPM was collected on Cambridge filter pads and eluted with DMSO to a stock concentration of 24 mg/mL for 3R4F and 50 mg/mL for the consumables.
- For the GVP generation, the gas phase that passed through the pad was collected by bubbling into 15 mL ice cold Ca^{2+} and Mg^{2+}-free PBS. A stock concentration of 50 mg (TPM equiv.)/mL was generated.

Ames Test

- Five strains (TA98, TA100, TA1535, TA1537 & TA102) were tested in the presence and absence of Aroclor 1254 induced rat S9.
- At least 5 concentrations per Test Article were tested according to OECD 471[4] and GLP. Three independent experiments were performed.

Mouse Lymphoma Assay (MLA)

- L5178Y cells were used and cells treated for 3h ±S9 and 24h –S9.
- The maximum concentration tested was limited by cytotoxicity (between 20-10% Relative Total Growth), or the maximum concentration achievable, or 2 mg/mL.
- At least 6 concentrations per Test Article were tested according to OECD 490[5] and GLP. Two independent experiments were performed.

Results

- In each *in vitro* assay, the vehicle and positive controls responded as per the historical control data and therefore the assays were considered valid.

Ames Test:

- 3R4F TPM consistently demonstrated statistically significant ($p \leq 0.05$) mutagenic activity in strains TA98, TA100 and TA1537 +S9 (Fig. 1). No mutagenicity was observed in any other strain ± S9.

- 3R4F GVP provided a weak mutagenic response ($p \leq 0.05$) in TA100 +S9 (Fig. 2). No mutagenicity was observed in any other strain ± S9.

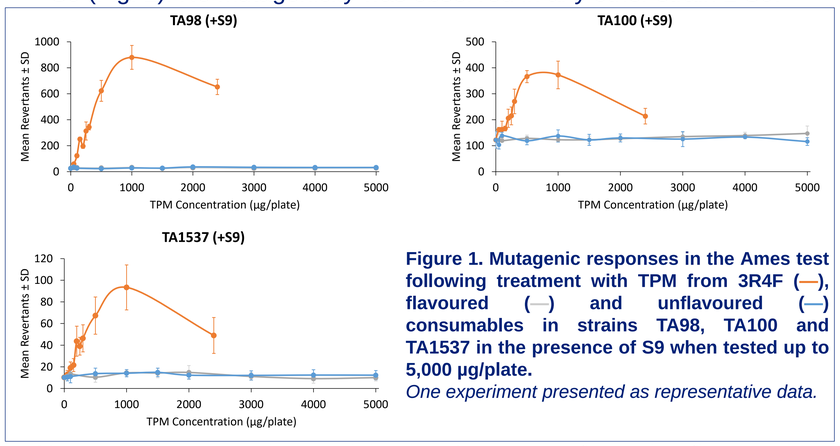

Figure 1. Mutagenic responses in the Ames test following treatment with TPM from 3R4F (—), flavoured (—) and unflavoured (—) consumables in strains TA98, TA100 and TA1537 in the presence of S9 when tested up to 5,000 µg/plate.
One experiment presented as representative data.

- No response was observed in the Ames test from the flavoured or unflavoured consumables TPM or GVP in any strain ±S9 (Fig. 1 & 2).

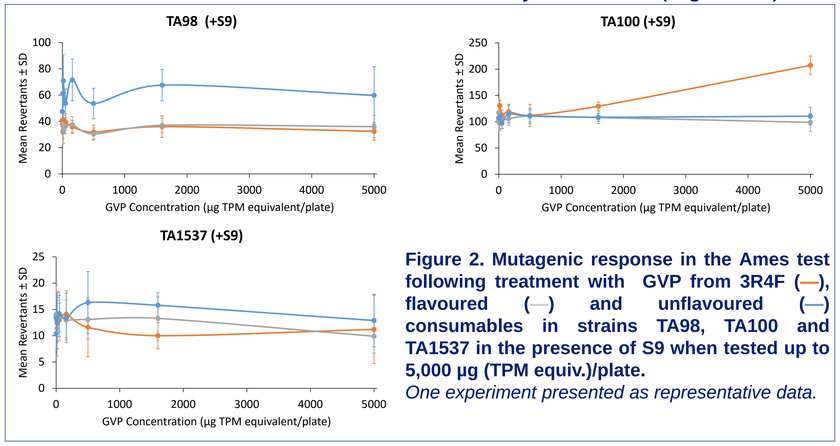

Figure 2. Mutagenic response in the Ames test following treatment with GVP from 3R4F (—), flavoured (—) and unflavoured (—) consumables in strains TA98, TA100 and TA1537 in the presence of S9 when tested up to 5,000 µg (TPM equiv.)/plate.
One experiment presented as representative data.

Mouse Lymphoma Assay

- The highest concentration analysed for mutation frequency (MF) was limited by excessive cytotoxicity of the Test Articles in these test conditions or the maximum concentration achievable.

- 3R4F TPM and GVP produced statistically significant ($p \leq 0.05$) increases in MF that were greater than the Global Evaluation Factor (GEF) (Fig. 3 & 4).

- The TPM from the flavoured and unflavoured consumables did not demonstrate any concentration related increases in MF and were significantly lower than the GEF (Fig. 3).

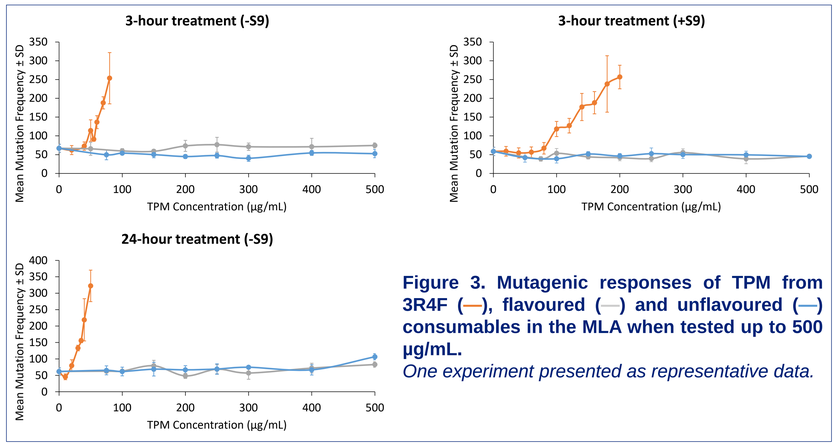

Figure 3. Mutagenic responses of TPM from 3R4F (—), flavoured (—) and unflavoured (—) consumables in the MLA when tested up to 500 µg/mL.
One experiment presented as representative data.

- Following GVP exposure, the flavoured and unflavoured consumables induced statistically significant increases in MF that were not statistically different from each other and the dose-response was lower than 3R4F (Figure 4).

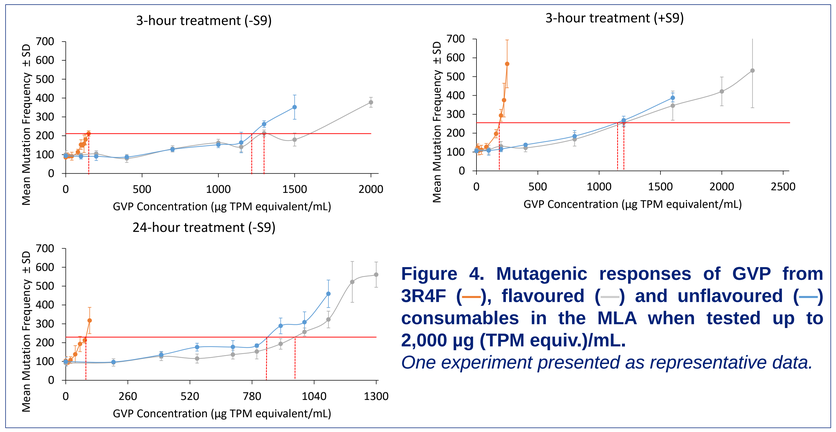

Figure 4. Mutagenic responses of GVP from 3R4F (—), flavoured (—) and unflavoured (—) consumables in the MLA when tested up to 2,000 µg (TPM equiv.)/mL.
One experiment presented as representative data.

Conclusions

- 3R4F TPM and GVP provided reproducible mutagenic responses in the Ames test (TA98, TA100 and TA1537 +S9; TA100 +S9 only with GVP) and in the MLA (all treatment conditions).
- The flavoured and unflavoured consumable TPMs did not provide evidence of mutagenicity in the Ames test or MLA up to the concentrations tested. Furthermore, no new mutagenic hazards were introduced.
- The GVP from both the flavoured and unflavoured consumables were not mutagenic in the Ames test.
- In the MLA, GVP from both consumables were mutagenic at concentrations over 5 times greater than those that induced a positive response with 3R4F.
- The addition of flavourings to the consumable that may thermally decompose did not add to the *in vitro* baseline responses of the unflavoured THP.
- The commercial product has also been tested in a range of *in vitro* assays[2].

References

[1]Forster, M., et al., 2015. *Chem Cent J., 9, 20.* 1
[2]Thorne, D, et al., 2018. Regul. Toxicol. Pharmacol., 93, 71-83
[3]Baker, R.R, et al., 2004. *Food Chem. Toxicol.* 42, 53–83.
[4]OECD Test Guideline 471, 1997.
[5]OECD Test Guideline 490, 2015.

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We have developed a prototype Tobacco Heating Product (THP) that heats a rod containing tobacco (consumable) up to 240°C to release nicotine, glycerol and flavouring components. To assess the hazards that such products may present and to determine whether any flavour ingredient thermal breakdown product may affect the baseline toxicity profile, the emissions *in vitro* toxicity were compared to a reference combustible tobacco product, 3R4F. The Total Particulate Matter (TPM) and Gas-Vapour Phase (GVP) of a THP with and without flavourings and 3R4F, were generated under Health Canada Intense smoking conditions. The TPM was captured on Cambridge filter pads and extracted with dimethyl sulfoxide and the GVP that passed through the pad was captured in ice-cold phosphate buffered saline. Both the TPM and GVP were tested in the Ames test (5 tester strains ±S9; OECD 471) and mouse lymphoma assay (L5178Y cells, treatments were conducted for 3h ±S9 and 24h -S9; OECD 490) and testing was conducted to Good Laboratory Practice.

In the Ames test, 3R4F TPM induced reproducible, statistically significant ($p \leq 0.05$), concentration-related increases in revertant colonies in tester strains TA98, TA100 and TA1537 +S9. The TPM from the flavoured and unflavoured THP did not induce any reproducible increases greater than the vehicle control in any strain or treatment condition. Following treatment with GVP from 3R4F and both THPs, there were no increases in revertants observed when tested up to 5,000 micrograms (TPM equivalent)/plate in any strain or treatment condition. The only exception to this was following treatment of 3R4F GVP in tester strain TA100 ±S9, where a small but reproducible statistically significant increase ($p \leq 0.05$) in revertants was observed at the highest concentrations.

In the mouse lymphoma assay, in each treatment condition, 3R4F TPM induced statistically significant ($p \leq 0.01$) increases in mutation frequency greater than the Global Evaluation Factor (GEF) and this was reproducible in two independent experiments. The TPM from both THP samples did not induce any increases that were concentration related or greater than the GEF and therefore were considered negative when tested up to 500 micrograms of TPM per millilitre. Following GVP exposure, 3R4F and both THP GVP samples induced statistically significant increases ($p \leq 0.01$) in mutation frequency that were concentration related and exceeded the GEF. However, the response from both THP GVP samples were comparable to each other and lower than the 3R4F GVP response.

This demonstrates that in the Ames test and mouse lymphoma assay, the emissions of a THP, in both TPM and GVP phases, is considerably less mutagenic and genotoxic than cigarette smoke and did not introduce any new hazards. The responses of the flavoured and unflavoured THPs were comparable demonstrating the absence of a flavour effect. Furthermore, the lower responses observed in these assays with the THPs is likely to be due to the lower heating temperature of the consumable, resulting in a chemically less complex emission, with fewer tobacco smoke constituents..