Reduced levels of harmful and potentially harmful constituents in heated tobacco aerosol translate to reduced *in vitro* (geno)toxicological outcomes

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1. BACKGROUND

Combustible tobacco smoking is a cause of serious disease in smokers including lung cancer, heart disease and emphysema[1,2]. Smoke generated from combustible cigarettes contains around 7000 chemicals, including harmful and potentially harmful constituents (HPHCs), which have been identified by a number of public health bodies[3,4]. However, next generation products (NGPs), such as heated tobacco products (HTPs), offer potentially reduced risk nicotine delivery, without the need for tobacco combustion, for adult smokers who would otherwise continue to smoke.

In the HTP category, nicotine delivery is achieved via heating of a reconstituted tobacco consumable (stick) (**Figure 1**) using a rechargeable device containing a heating element (**Figure 2**) to generate an aerosol which is inhaled by the adult smoker. This heating occurs at temperatures below those required for combustion. The aerosols generated from HTPs have been demonstrated to contain fewer and substantially reduced levels of HPHCs compared to cigarette smoke[5,6]; this reduction in measured HPHCs correlates with reduced *in vitro* toxicological outcomes[5,7].

This study aimed to compare the aerosol chemistry of a prototype HTP (p-HTP) to the composition of 1R6F Reference Cigarette smoke using a selected list of HPHCs. The effects of the smoke and aerosol were then compared in a number of *in vitro* toxicological endpoints related to genotoxicity.

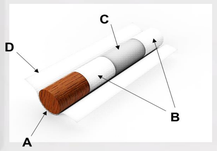

Figure 1: Image representative of the reconstituted tobacco p-HTP stick used in this study, consisting of reconstituted tobacco (**A**), filters (**B**), a cardboard tube (**C**) and outer paper (**D**).

Figure 2: Rendered image representative of the p-HTP device used in this study with a reconstituted tobacco stick inserted.

2. METHODS

2.1. Smoke/ aerosol analysis

Levels of selected HPHCs within 1R6F Reference Cigarette (University of Kentucky, Center for Tobacco Reference Products) smoke and p-HTP aerosol were measured, including the WHO TobReg 9 list of analytes[4] and nicotine.

Table 1: Stick conditioning and smoking regimes used in this study.

Product	Stick conditioning	Smoking regime
1R6F Reference Cigarette	ISO 3402 (at least 48h at 22±1°C and 60±3% relative humidity)	ISO 20778 (55ml puff volume, 2s puff duration, 30s puff interval, bell shaped puff profile, ventilation blocking)
p-HTP	ISO 3402	ISO 20778 (modified, e.g., no ventilation blocking)

2.2. *In vitro* assessment

Table 2: Details of *in vitro* assessments used in this study. ALI: air-liquid interface; SAEIVS: Smoke Aerosol Exposure *In Vitro* System.

Assay	Cells	Exposure method	Output
Neutral red uptake (NRU) (cytotoxicity)	Beas-2B (ECACC 95102433)	ALI using the SAEIVS 1R6F: 0-0.6 puffs p-HTP: 0-27 puffs	Number of puffs required to induce 50% cytotoxicity (EC_{50})
Micronucleus (MN)	V79 (+/-S9) (ECACC 86041102)	ALI using the SAEIVS 1R6F: 0-2.5 puffs +S9; 0-1.67 puffs -S9 p-HTP: 0-36 puffs +/-S9	Number of puffs required to increase micronucleus frequency to 3x above background levels (ECMN3)
Ames	*Salmonella typhimurium*: TA98, TA100 (+/-S9) (Trinova Biochem GmbH)	Fresh smoke/ aerosol generated using the Vitrocell VC 10-S Smoking Robot and bubbled through bacterial suspensions (increasing puff-wise concentrations)	Slope of response compared to background
High content screening (HCS)	Normal human bronchial epithelial (NHBE) (PromoCell GmbH)	Exposures to increasing concentrations (for 4h or 24h) of 1R6F smoke or p-HTP aerosol bubbled PBS (bPBS) generated using the Vitrocell VC 10-S. Bubbling was consistent with the methods used by Czekala *et al.*[8]	Cells stained for seven markers related to cell stress and screening carried out using the Thermo Scientific ArrayScan XTI High Content Analysis Reader. Endpoints detailed in **Table 3**

Table 3: HCS endpoints assessed in this study and the markers used.

Marker	Endpoint
Cell count	Cytotoxicity
Cytochrome c release	Mitochondrial stress
Glutathione (GSH) levels	Oxidative stress
Phosphorylation of H2AX (γH2AX)	DNA damage
Phosphorylation of c-jun	Cell cycle control
NFκB translocation	Cellular stress response
Mitochondrial membrane potential (MMP)	Mitochondrial dysfunction

Relevant positive controls were also tested in parallel. The responses in the above *in vitro* assays were calculated as fold-changes compared to 1R6F. Statistical analyses were carried out using GraphPad PRISM version 8.4.3.

3. RESULTS

3.1. Reduced levels of HPHCs in p-HTP aerosol

- HPHC levels were substantially reduced in the p-HTP aerosol compared to 1R6F smoke per puff (**Figure 3**)
- Around 43% of the nicotine found in one puff of 1R6F smoke was in one puff of the p-HTP aerosol. However, even when normalised for nicotine concentration (i.e., 1 puff of 1R6F smoke and 2.3 puffs of p-HTP aerosol), levels of the HPHCs in the p-HTP aerosol were still substantially reduced

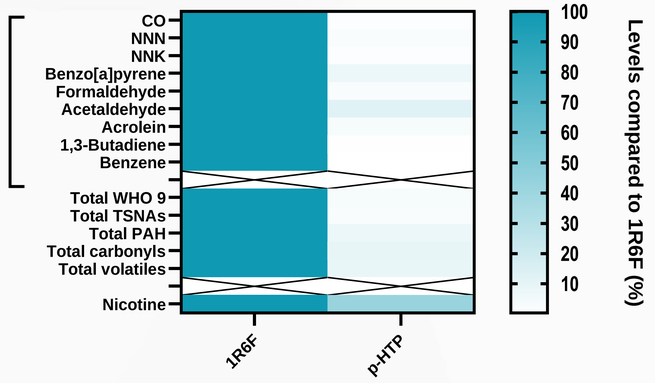

Figure 3: Heatmap indicating levels of selected analytes in 1R6F Reference Cigarette smoke and the p-HTP aerosol per puff, including the WHO TobReg 9 analytes, totals for the chemical classes measured and nicotine, on a per puff basis. NNN = N-nitrosonornicotine; NNK = nicotine-derived nitrosamine ketone; TSNAs = tobacco specific nitrosamines; PAH = polycyclic aromatic hydrocarbon.

3.2. Reduced levels of HPHCs translate to reduced *in vitro* toxicity

- *NRU and micronucleus assays*: the effects of p-HTP aerosol were substantially reduced compared to 1R6F smoke (**Table 4**, **Table 5**, **Figure 4**)
- *Ames test*: p-HTP aerosol was not mutagenic under the conditions of the test; 1R6F was classed as mutagenic (**Table 6**, **Figure 4**)
- *HCS*: compared on a nicotine concentration basis, NHBE cell responses to p-HTP aerosol were substantially reduced compared to 1R6F (**Figure 5**)

Table 4: NRU assay (detailed in **Table 2**) results.

Product	EC_{50} (puffs)	Fold-change compared to 1R6F
1R6F	0.234	1
p-HTP	9.14	39

Table 5: Micronucleus assay (detailed in **Table 2**) results.

Product	ECMN3		Fold-change compared to 1R6F	
	+S9	-S9	+S9	-S9
1R6F	1.6	1.3	1	1
p-HTP	12.6	16.6	8.0	12.8

Table 6: Ames test (detailed in **Table 2**) results.

Product	TA98: response slope (significantly changed compared to 0?)		TA98: fold-change compared to 1R6F		TA100: response slope (significantly changed compared to 0?)		TA100: fold-change compared to 1R6F	
	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
1R6F	2.00 (yes)	0.826 (yes)	1	1	2.74 (yes)	1.702 (yes)	1	1
p-HTP	-0.00494 (no)	0.0124 (no)	405.0	66.9	0.106 (no)	0.148 (no)	25.9	11.5

Figure 4: Heatmap indicating effects of p-HTP aerosol compared to 1R6F smoke (maximal response (100%)) in the NRU, micronucleus and Ames assays, based on the data in **Tables 4, 5** and **6.**

Figure 5: ToxPi visual plots of HCS data outputs at a low nicotine concentration (1.75µg/ml = 0.019puffs/ml for 1R6F), at an equivalent (7±0.3µg/ml = approx. 0.075puffs/ml for 1R6F and 0.18puffs/ml for the p-HTP) and a high nicotine concentration (17±0.9µg/ml = 0.45puffs/ml for the p-HTP). Plotted fold changes in responses (compared to the endpoints' respective background levels (set to 1-fold)) are scaled according to the maximum values observed for each endpoint (over both 4h and 24h) across all doses and samples tested (note, the highest fold responses (in either direction) were always induced by 1R6F). The key plot on the left indicates which slices correspond to which endpoint and timepoint.

4. CONCLUSIONS

The levels of HPHCs analysed within the p-HTP aerosol were substantially reduced compared to 1R6F Reference Cigarette smoke. When normalised on a nicotine basis, these reductions were still substantial. This reduction translated into reduced *in vitro* toxicological outcomes in the NRU, micronucleus and Ames tests. Further to this, when NHBE cells were exposed to smoke/ aerosol bPBS, and exposures compared on a nicotine delivery basis, responses to the p-HTP were greatly reduced compared to 1R6F. The assessment of multiple *in vitro* endpoints related to genotoxicity/ cellular stress provides a greater pre-clinical understanding of the effects of the test products. Overall, these results are consistent with the scientific evidence[5,6,7] and therefore add to the weight of evidence that HTPs may offer potentially reduced harm nicotine delivery to adult smokers who would otherwise continue to smoke.

[1]International Agency for Research on Cancer (2012). "Personal habits and indoor combustions. A review of human carcinogens: IARC monographs on the evaluation of carcinogenic risks to humans." Volume 100E https://monographs.iarc.fr/wp-content/uploads/2018/06/mono100E.pdf (Last accessed 13-02-2021). [2]United States Surgeon General (2010). "Surgeon General's Report – How tobacco smoke causes disease: The biology and behavioural analysis for smoking-attributable disease." https://www.cdc.gov/tobacco/data_statistics/sgr/2010/index.htm (Last accessed 13-02-2021). [3]FDA (2011). "Guidance for Industry and FDA Staff. In: "Harmful and Potentially Harmful Constituents" in Tobacco Products as Used in Section 904(e) of the Federal Food, Drug, and Cosmetic Act, 2011 (Revised)." https://www.fda.gov/downloads/TobaccoProducts/Labeling/RulesRegulationsGuidance/UCM241352.pdf. [4]Burns, D., Dybing, E., Gray, N., Hecht, S., Anderson, C., Sanner, T., O'Connor, R., Djordjevic, M., Dresler C., Hainaut, P., Jarvis, M., Opperhuizen, A., Straif, K. (2008). "Mandated lowering of toxicants in cigarette smoke: a description of the World Health Organization TobReg proposal." Tobacco Control 17: 132-141. DOI: 10.1136/tc.2007.024158. [5]Schaller, J.-P., Keller, D., Poget, L., Pratte, P., Kaelin, E., McHugh, D., Cudazzo, G., Smart, D., Tricker, A., Gautier, L., Yerly, M., Reis Pires, R., Le Bouhellec, S., Ghosh, D., Hofer, I., Garcia, E., Vanscheeuwijck, P., Maeder, S. (2016). "Evaluation of the