The *In Vitro B*iological Assessment of a Tobacco Heating Product and Comparison With a Cigarette Smoke

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Introduction

Tobacco heating products (THPs) represent a subset of the next-generation nicotine and tobacco product category, in which tobacco is heated at temperatures of less than 350°C instead of burning (900°C), having the potential to significantly reduce cigarette smoke toxicants. THPs hold great potential for reducing the harm associated with tobacco use, but this needs to be scientifically proven.

The aim of this study was to characterise the aerosol emissions and assess the biological impact of the novel THP; THP1.0 (commercially known as glo™) (**Figure 1**), comparing results to a reference 3R4F cigarette.

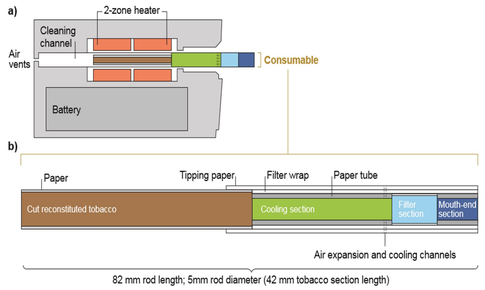


Figure 1. Schematic drawing and picture of THP1.0 with a tobacco consumable[1].

Materials and Methods

Assessment of emissions

The emissions of toxicants in THP1.0 aerosol were compared with those from a reference 3R4F cigarette under a machine-puffing regimen of 55 mL puff volume, 2 s puff duration and 30 s puff interval[1]. The list of toxicants measured included those proposed by Health Canada, the WHO Study Group on Tobacco Product Regulation (TobReg), the US Food and Drug Administration and possible thermal breakdown products. Overall, 22 different analytical techniques were used to quantify the emissions of 126 analytes in 3R4F mainstream smoke and THP1.0 emissions, as described in Forster et al., 2018[2].

In vitro assessment

Using the same puffing regimen as described above, three different test matrices were generated for *in vitro* assessment:

- Total Particulate Matter (TPM) – trapped on a Cambridge filter pad and eluted at 24 mg/mL in DMSO. TPM is diluted in cell culture medium to treat cells in submerged culture.
- Whole aerosol (WA) – cells directly exposed to aerosol at the air-liquid interface.
- Aerosol aqueous extract (AqE) – aerosol bubbled through cell culture medium in an impinger. AqE is diluted in cell culture medium to treat cells in submerged culture.

Neutral red uptake (NRU) cytotoxicity assay

TPM cytotoxicity was assessed using BALB/c 3T3 mouse fibroblasts3. WA cytotoxicity was assessed using human bronchial epithelial cells (H292) exposed at the air-liquid interface for 1 h at dilutions of 1:20-1:10,000 for 3R4F and 1:2- 1:200 for THP1.0 (aerosol:air; v:v)[3].

Luciferase-based reporter gene assay to assess oxidative stress

Antioxidant response element (ARE) transcriptional activation in stably transfected H292 cells were assessed after 6 and 24 h treatment[4].

Endothelial wound healing (scratch) assay

Artificial wounds were created in monolayers of human umbilical vein endothelial cells (HUVEC). Cells were treated with AqE, and wound repair was assessed over 22 hours using image analysis, as previously reported[5].

Multiparametric analysis using high-content screening (HCS) approaches

The Cellomics Arrayscan VTi platform was used to assess 10 endpoints in normal human bronchial epithelial cells (NHBEs) after 4 or 24 h exposures, as previously described[4].

Results

Quantification of FDA priority toxicants

Toxicant levels in the emissions from THP1.0 were significantly lower than those from 3R4F (Table 1).

Table 1. 3R4F reference cigarette MSS yields and THP1.0 emission yields for the 18 priority constituents in the US FDA abbreviated list presented on a consumable basis. Values calculated using replicate data per analyte (N = 5)

Parameter	Unit	3R4F	THP1.0	
		Mean per Consumable	Mean per Consumable	%Red[n] per Consumable
1,3-Butadiene	µg	108	BDL (0.029)	>99.9
Acetaldehyde	µg	2200	111	95.0
Acrolein	µg	157	2.22	98.6
Benzene	µg	78.6	NQ (0.056)	>99.9
Benzo[*a*]pyrene	ng	12.9	NQ (0.354)	97.7
Carbon Monoxide	mg	32.0	NQ (0.223)	99.8
Formaldehyde	µg	54.10	3.29	93.9
4-(*N*-Nitrosomethylamino)-1-(3-pyridyle)-1-butanone (NNK)	ng	281	6.61	97.7
Nitrosonornicotine (NNN)	ng	263	24.7	90.6
			Average	97.0

In vitro assessment

WA cytotoxicity assessment demonstrated that 3R4F produced a concentration-related decrease in cell viability, resulting in complete cytotoxicity at the top concentrations tested. THP1.0 induced significantly less cytotoxicity at comparable and higher levels of nicotine delivered to the cells, Figure 2.

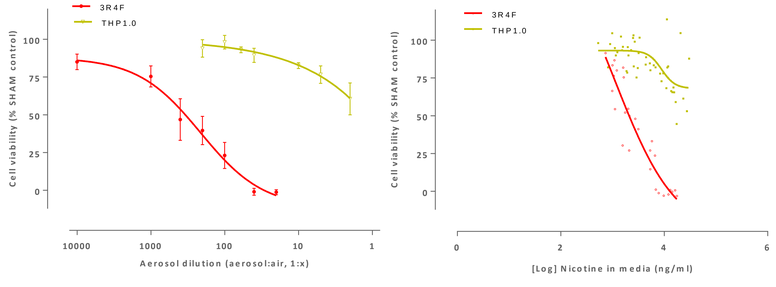


Figure 2. Neutral red uptake determined cell cytotoxicity of H292 cells after 1 hour exposure to a range of dilutions of the two test articles generated on the Borgwaldt RM20S smoking machine. Cell cytotoxicty is expressed as a function of (a) aerosol dilution, and (b) nicotine levels measured in the media following exposure.

3R4F was positive in 4 endpoints assessed using a HCS approach (Table 2). THP1.0 was negative for each HCS endpoint, apart from activation of the antioxidant response element (ARE), where there was a moderate response at both the 4 and 24 h timepoints. However, the data showed a significantly higher response to TPM generated from 3R4F than from THP1.0 at both timepoints tested, Figure 3.

Table 2. High content screening assay

Endpoint	Exposure time (h)	3R4F	THP1.0
ATP	4	60*	-
	24	120	-
Cell count	4	-	-
	24	-	-
Glutathione content	4	120	-
	24	-	-
Mitochondrial mass	4	-	-
	24	-	-
Mitochondrial membrane potential	4	120	-
	24	-	-
Nuclear size	4	-	-
	24	-	-
ROS formation	4	-	-
	24	-	-
DNA structure	4	-	-
	24	-	-
DNA damage (p-H2AX)	4	-	-
	24	60*	-
Stress kinase (p-c-Jun)	4	-	-
	24	-	-

Values are the minimum required TPM concentration (µg/mL) to elicit a ≥ 1.5-fold increase in assay signal from the 0.5% DMSO vehicle control or a 30% decrease in signal for the ATP, cell count, glutathione content, mitochondrial mass or mitochondrial membrane potential assay endpoints. Stars indicate a TPM-concentration dependant response.

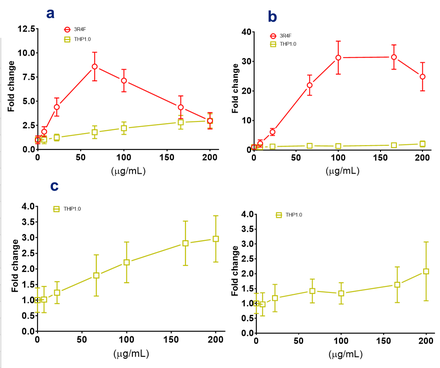
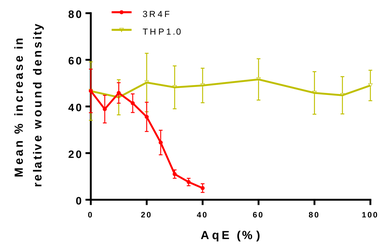


Figure 3. Activation of the H292-ARE-Luc2P RGA following exposure to 3R4F and THP1.0. Data shown are mean fold changes in response normalized to the vehicle control (0.83% DMSO). Activation following (a) 6h exposure to 3R4F and THP1.0 (b) 24h exposure to 3R4F and THP1.0 (c) 6h exposure to THP1.0 (d) 24hr exposure to THP1.0.

3R4F inhibited wound repair in a HUVEC monolayer, in a dose-dependant manner. AqE from THP1.0 did not significantly affect wound repair, up to the maximum concentration tested (100% AqE; Figure 4).

Figure 4. Wound healing rates in HUVEC monolayers during 24 hour treatment with AqE from 3R4F and THP1.0. Data are mean ± S.D. (n=6).



Conclusions

- The tobacco heating product (THP1.0) showed little or no activity in any of the *in vitro* assays where it was assessed.
- Activity was significantly less than from 3R4F reference cigarette across all studies.
- These studies indicate that this novel product has the potential to confer reduced risk of disease compared to cigarette smoking.

References

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