The pre-clinical assessment of a tobacco heating product (glo^{TM})

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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.

Objective

To characterise the aerosol emissions and assess the biological impact of the novel THP; THP1.0 (commercially known as glo^{TM}) (**Figure 1**), comparing results to a reference 3R4F cigarette.

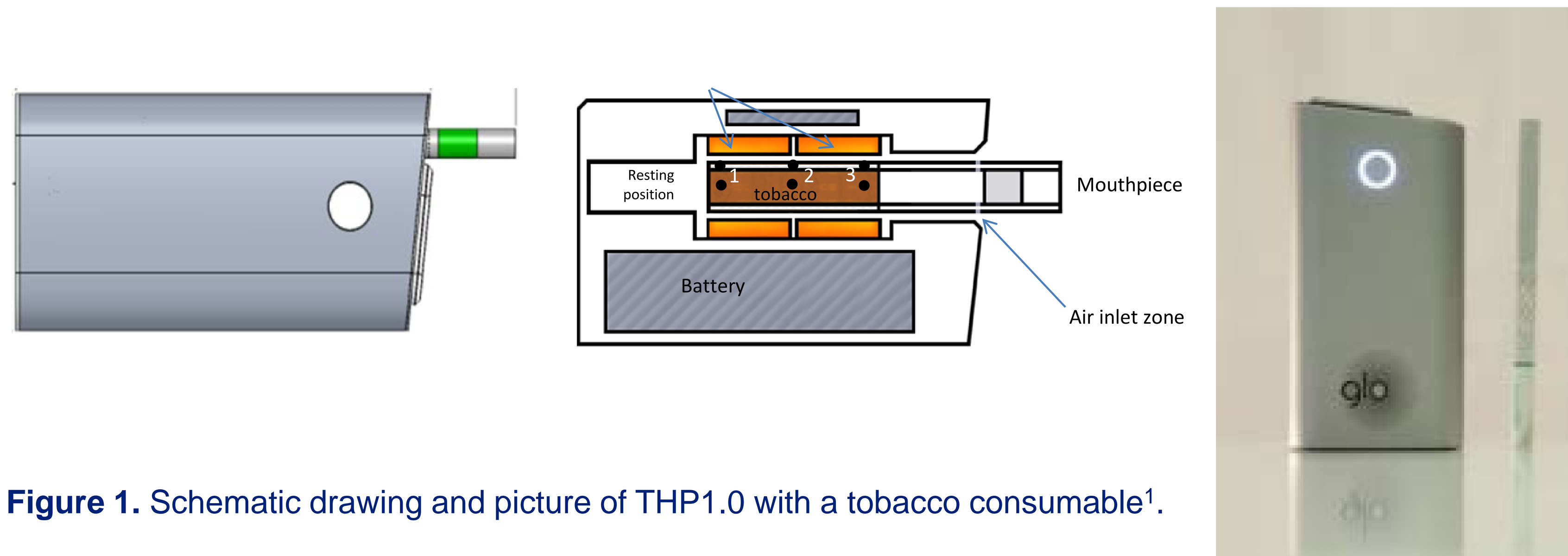

Figure 1. Schematic drawing and picture of THP1.0 with a tobacco consumable[1].

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. The list of toxicants measured included those proposed by Health Canada, the WHO Study Group on Tobacco Product Regulation (TobReg), the US FDA 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.,* 2017.

In vitro assessment

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

Total Particulate Matter (TPM)**:** Approximately 150 mg of TPM was collected on 44 mm Cambridge filter pads (Whatman, UK). DMSO (Sigma-Aldrich, UK) was used to elute the TPM from the pads to a stock concentration of 24 mg/mL.

Whole aerosol (WA): A Vitrocell VC10® smoking robot (Vitrocell Systems, Germany) was used to generate whole aerosols for the Ames assay, as previously described[3]. A Borgwaldt RM20S exposure system was used for the cytotoxicity assay, as detailed previously[4].

Ames bacterial reverse mutation assay

TPM exposures were conducted to the principles of OECD 471, using five *S. typhimurium* strains: TA98, TA100, TA1535, TA1537 and TA102 ± metabolic activation (S9). For product WA exposures, the Ames assay was employed with *S. typhimurium* tester strains TA98, TA100, TA1535, TA97 and TA102 using a modified methodology as previously described[3].

Mouse lymphoma assay (MLA)

TPM was assessed following OECD 490, ± S9 with short 3 h exposures and longer 24 h –S9 exposures[3].

Neutral red uptake (NRU) cytotoxicity assay

TPM cytotoxicity was assessed using BALB/c 3T3 mouse fibroblasts[3]. WA cytotoxicity was assessed using human bronchial epithelial cells (H292) exposed at the air-liquid interface (ALI) 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)[4].

Bhas cell transformation assay

The potential of TPM from the products to induce tumour development was evaluated using the Bhas 42 cell transformation assay, promoter protocol[3]. TPM was tested at various concentrations up to a maximum concentration of 48 µg/mL.

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[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[5].

Results

Quantification of FDA priority toxicants

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

Parameter	Unit	3R4F	THP1.0	
		Mean per Consumable	Mean per Consumable	%Red[a] 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

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)

The data for 126 chemicals including the extended FDA, Health Canada and WHO list can be found in Forster *et al.*, 2017.

In vitro assessment

THP1.0 demonstrated significantly reduced toxicological response compared to 3R4F in genotoxicity, cytotoxicity and cell transformation assays, **Table 2**. THP1.0 was negative across all assays, under each of the conditions tested. In contrast, 3R4F was positive for each endpoint.

Table 2. Regulatory toxicity testing: ***in vitro*** **cytotoxicity, mutagenicity and tumour promotion.**

Treatment condition	NRU[1] TPM	AMES[2] TPM		AMES[3] WA	MLA[4] TPM			Bhas[5] TPM
	-S9	-S9	+S9	+S9	3h – S9	3h+ S9	24h- S9	-S9
3R4F	+	+ (TA98, TA100)	+ (TA98, TA100, TA1537)	+ (TA98, TA100)	+	+	+	+
THP1.0	-	-	-	-	-	-	-	-

TPM= total particulate matter
WA= whole aerosol
1= tested up to 240 µg/mL over 24 h
2= tested up to 2400 µg/mL over 72 h plate incorporation and preincubation
3= tested to equivalent doses using QCM technology
4= tested up to 240 µg/mL +/-S9 over 3 treatment conditions
5= tested up to 120 µg/mL for 10 days

3R4F was positive for each endpoint assessed using a HCS approach. In all but two endpoints 3R4F was positive at both timepoints tested, **Table 3**. 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 3. High content screening

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.

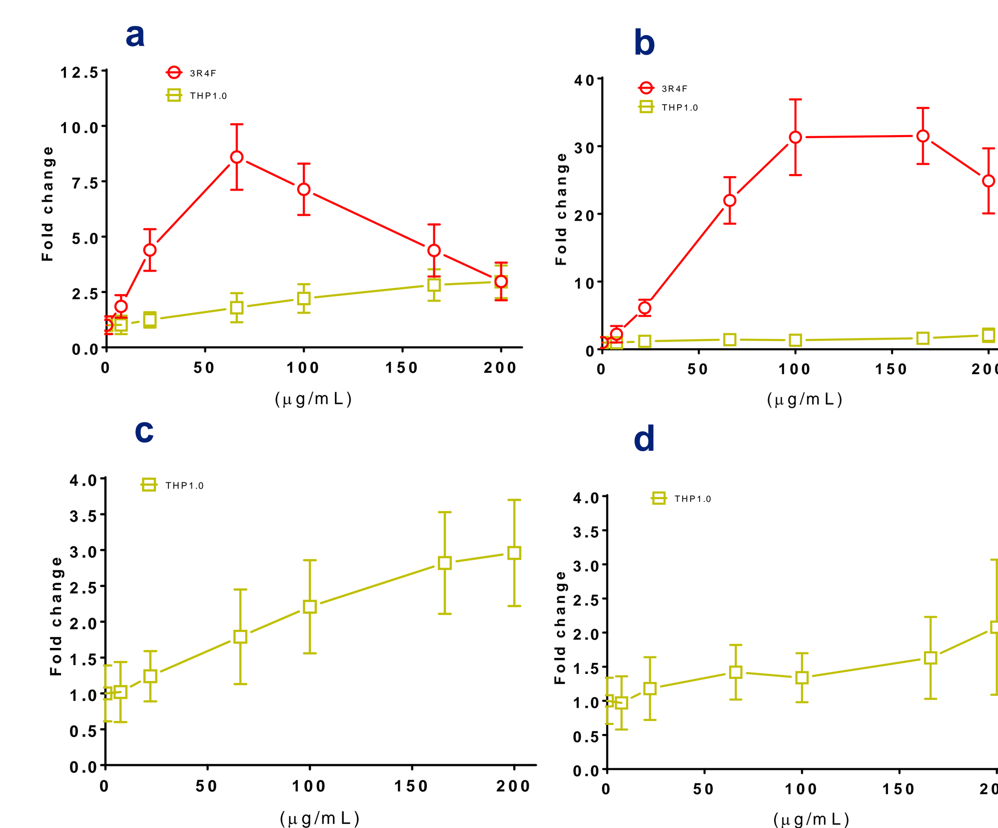

Figure 3. Activation of the H292-ARE-Luc2P RGA following exposure to 3R4F and THP1.0. Data shown are mean fold changes in response normalized to the vehicle control (0.83% DMSO). Activation following (a) 6h exposure to 3R4F and THP1.0 (b) 24h exposure to 3R4F and THP1.0 (c) 6h exposure to THP1.0 (d)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

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 4.**

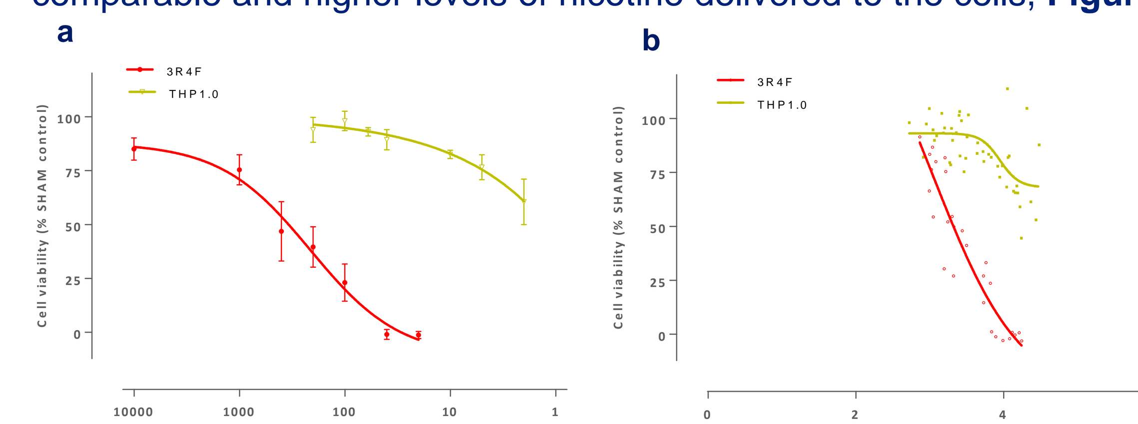

Figure 4. Neutral red uptake determined cell cytotoxicity of H292 cells after 1 hour exposure to a range of dilutions of the two test articles generated on the Borgwaldt RM20S smoking machine. Cell cytotoxicity is expressed as a function of (a) aerosol dilution, and (b) nicotine levels measured in the media following exposure.

Conclusions

- Toxicant levels in THP1.0 emissions were significantly reduced across all chemical classes compared to 3R4F reference cigarette
- Across all the *in vitro* techniques employed a clear positive response was observed with 3R4F cigarette smoke particulate matter and whole aerosol
- THP1.0 particulate matter and whole aerosol showed little or no activity in any of the *in vitro* assays at doses equivalent or higher than 3R4F reference cigarette
- The data generated add to growing evidence that suggests THPs may provide a less risky alternative to traditional cigarettes, however further studies investigating the longer terms effects on consumers is required to substantiate disease relevant risk reduction

References

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