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Assessment of tobacco heating product THP1.0. Part 5: *In vitro* dosimetric and cytotoxic assessment

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ABSTRACT

Tobacco heating products (THPs) represent a subset of the next-generation nicotine and tobacco product category, in which tobacco is typically heated at temperatures of 250–350 °C, thereby avoiding many of the harmful combustion-related toxicant emissions of conventional cigarettes. In this study, we have assessed aerosol generation and cytotoxicity from two commercially available THPs, THP1.0 and THS, relative to tobacco smoke from 3R4F reference cigarettes, using an adapted Borgwaldt RM20S Smoking Machine. Quantification of nicotine in the exposed cell-culture media showed greater delivery of nicotine from both THPs than from the cigarette. Using Neutral Red Uptake assay, THPs demonstrated reduced *in vitro* cytotoxicity in H292 human bronchial epithelial cells as compared with 3R4F cigarette exposure at the air–liquid interface ($p < 0.0001$). Both THPs demonstrated a statistically similar reduction in biological response, with >87% viability relative to 3R4F at a common aerosol dilution (1:40, aerosol:air). A similar response was observed when plotted against nicotine; a statistical difference between 3R4F and THPs ($p < 0.0001$) and no difference between the THPs ($p = 0.0186$). This pre-clinical *in vitro* biological testing forms part of a larger package of data to help assess the safety and risk reduction potential of next-generation tobacco products relative to cigarettes, using a weight of evidence approach.

1. Introduction

Cigarette smoking is one of the primary causes of preventable death and has been linked with the development of smoking-related diseases, such as cardiovascular disease, chronic obstructive pulmonary disease and lung cancer (US DHHS, 2014). Over the past decade, there has been an increase in the availability of next-generation tobacco products (NGPs) including vapour products, such as electronic-cigarettes (e-cigarettes) and tobacco heating products (THPs) that heat rather than combust tobacco. Most NGPs function by heating either tobacco (Eaton et al., 2017) or a nicotine-containing liquid (Margham et al., 2016) to produce a nicotine-containing aerosol comprising significantly lower levels of cigarette smoke-related toxicants (Forster et al., 2017; Margham et al., 2016; Poynton et al., 2017; Schaller et al., 2016).

An international panel of experts in public health and tobacco use studied the available published science to assess the risk profile of a range of tobacco and nicotine products (Nutt et al., 2014). Based on their findings and a review of the published literature, Public Health England reported that e-cigarettes were likely to be 95% less harmful than conventional cigarettes (McNeill et al., 2015). These conclusions were further supported by reports from the Royal College of Physicians (RCP, 2016) and Cancer Research UK (Cancer Research UK, 2017). Another recent study from Shahab et al., (2017), reported that after 6 months of e-cigarette use, reduced levels of biomarkers of exposure were maintained relative to cigarette use. Further recent research has shown that e-cigarette emissions contain lower levels of toxicants as compared with cigarette smoke (Goniewicz et al., 2014; Margham et al., 2016) and considerably reduced biological effects in a range of laboratory tests relative to cigarettes (Azzopardi et al., 2016; Banerjee et al., 2017; Fields et al., 2017; Misra et al., 2014; Taylor et al., 2016; Thorne et al., 2016, 2017).

THPs are less-well studied than e-cigarettes, largely due to their current limited availability in markets worldwide. Whereas e-cigarettes have e-liquid as a source of nicotine, THPs use tobacco as the nicotine source. THPs and hybrid tobacco products typically heat tobacco to temperatures of 250–350 °C or <30 °C, respectively,

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Abbreviations

ALI	air-liquid interface
HCI	Health Canada Intense cigarette smoking regime
HCI_m	modified Health Canada Intense smoking regime
NGP	next generation tobacco and nicotine product
NRU	Neutral Red Uptake
RM20S	Borgwaldt RM20S Smoking Machine
THP	Tobacco Heating Product
THP1.0	Tobacco Heating Product version 1
THS	Tobacco Heating System
3R4F	University of Kentucky reference cigarette

which are significantly lower than the temperatures of ~900 °C found during the combustion of tobacco in cigarettes, thus avoiding many of the harmful toxicant emissions (Breheny et al., 2017; Eaton et al., 2017). At heating temperatures <350 °C, nicotine is still released in a condensation aerosol, but with much fewer particle and vapour phase toxicants, and those that are still delivered in the aerosol are present at much lower concentrations, up to 90% reductions relative to smoke from conventional tobacco products (Eaton et al., 2017; Forster et al., 2017; Schaller et al., 2016). Additional studies have demonstrated that, as compared with a 3R4F reference cigarette, THPs have significantly reduced levels of harmful and potentially harmful constituents (Doolittle et al., 1990; Forster et al., 2017; Foy et al., 2004; Smith et al., 2016), resulting in reduced toxicity in laboratory-based *in vitro* tests (Doolittle et al., 1990; Foy et al., 2004; Munakata et al., 2017; Schaller et al., 2016).

Pre-clinical *in vitro* biological testing can help generate data to support a weight-of-evidence approach in assessing the risk reduction potential of NGPs (Lowe et al., 2015; Murphy et al., 2017; Smith et al., 2016). *In vitro* test systems are used to assess the genotoxicity and mutagenicity of tobacco smoke extracts, including particulate matter extracts, aqueous extracts and whole aerosols (Ishikawa and Ito, 2017; Kuehn et al., 2015; Munakata et al., 2017; Schaller et al., 2016; Thorne et al., 2015, 2016). With recent advances in cell-culture and aerosol exposure systems, more physiologically relevant *in vitro* assessments in which lung cells are exposed to aerosols at the air-liquid interface (ALI) can be used to evaluate the biological effects of NGPs (Azzopardi et al., 2016; Banerjee et al., 2017; Fields et al., 2017; Gonzalez-Suarez et al., 2016; Iskandar et al., 2017; Schaller et al., 2016; Smith et al., 2016; Taylor et al., 2016). To ensure appropriate and reliable *in vitro* biological assessment of aerosols, both the exposure system used to generate aerosols and the generated aerosol should be characterised appropriately to understand and verify the dose delivered. Dosimetry tools enable the characterisation of *in vitro* exposure systems, such as the Vitrocell VC10 (Vitrocell Systems GmBH, Waldkirch, Germany) and the Borgwaldt RM20S Smoking Machine (Borgwaldt KC GmbH, Hamburg, Germany), which are commonly used to generate, dilute and deliver aerosols from tobacco and NGPs to cell cultures maintained in exposure chambers. These and similar exposure systems have been extensively evaluated by various dosimetry tools to investigate exposure system characteristics such as aerosol transit, deposition and losses (Adamson et al., 2011, 2016, 2017; Ishikawa et al., 2016; Li, 2016; Majeed et al., 2014; Mülhopt et al., 2009; Scian et al., 2009; Steiner et al., 2016; Thorne and Adamson, 2013). Knowledge and maintenance of the accuracy and precision of aerosol delivery to cellular cultures in exposure chambers is essential to ensure a reliable and reproducible generation of aerosols, and in turn, more robust biological data (Kaur et al., 2010).

In this study, building on our current knowledge of *in vitro* exposure systems and NGP aerosols, we have assessed aerosol generation and cytotoxicity from two commercially available THPs, THP1.0 and THS (Fig. 1), and compared the findings with those from tobacco smoke from 3R4F reference cigarettes (Fig. 1). We have used an adapted Borgwaldt RM20S Smoking Machine to generate, dilute and deliver THP aerosols to *in vitro* cultures (Fig. 2). As quality control for the exposure system, we monitored syringe precision and accuracy using hydrocarbon analysis, and plotted control charts of dilution precision and accuracy throughout the study. Using nicotine analysis, we assessed the aerosols generated during the study to enable accurate quantification and dosimetric characterisation of the products being evaluated. Puff-by-puff nicotine was measured at the source of aerosol generation to understand the profile of test articles, and was compared with target analytical values previously obtained; nicotine was also quantified in the cell culture media after exposure. We then investigated the cytotoxic potential of these products using an *in vitro* human bronchial epithelial cell line (NCI-H292), exposed for 1 h at the ALI over a series of aerosol dilutions, and compared the findings with those from 3R4F reference cigarette smoke. Cytotoxicity was measured using the Neutral Red Uptake (NRU) assay as described previously (Azzopardi et al., 2015).

2. Materials and methods

The biological response – cell death/decreased proliferation – was measured by an NRU assay, and the dosimetry of each cellular exposure was characterised by quantifying nicotine in the exposed cell culture media. In addition, consideration was given to the Borgwaldt RM20S Smoking Machine (Fig. 2), and repeatability of aerosol generation and aerosol dilution precision and accuracy were assessed and verified before any biological exposure experiment was conducted.

2.1. Test articles, aerosol generation and exposure parameters

The Borgwaldt RM20S Smoking Machine (serial number 0508,432) and associated cell culture exposure chambers used in this study have been previously described (Adamson et al., 2011; Azzopardi et al., 2015, 2016; Maunders et al., 2007; Phillips et al., 2005). Two sets of syringes were used for each of the eight syringe positions (A-H); seven positions were for diluting test article aerosol and one position (the same one) was always reserved as the air control (sham). There were three test articles in this study: a reference cigarette and two THPs (Fig. 1). Reference cigarettes (3R4F, University of Kentucky) were smoked for 1 h at the Health Canada Intense (HCI) regime: 55 ml puff over 2 s, every 30 s, with filter vents blocked. Two commercially available THPs available in Japan, THP1.0 (Eaton et al., 2017) and THS (Schaller et al., 2016; Smith et al., 2016), were tested at the same puffing regime in comparison to the 3R4F reference cigarette. All devices and consumables were sourced from Japan. Complete product details for the THP1.0 and THS device, consumable and emission chemistry data are described in Eaton et al. (2017) and Schaller et al. (2016), respectively.

THPs were button-activated to initiate device heating prior to syringe activation. THP1.0 was activated 40 s prior to puffing and each consumable was puffed 8 times before being replaced; THS was activated 30 s prior to puffing and each consumable was puffed 12 times before being replaced. Different heat cycles and consumable puff numbers were mandated by the product design specification for each THP, as described in the manufacturers' usage instructions. The use of a bespoke NGP switch rig (dual linear

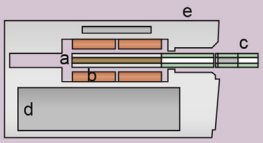
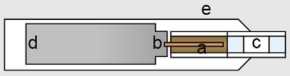
Code	Product type and manufacturer	Temperature (°C) of consumable during operation	Aerosol formation mechanism	Reference
3R4F	**Cigarette:** 3R4F scientific reference cigarette, University of Kentucky a. Tobacco b. Filter c. Paper 	>900	Pyrolysis and combustion of tobacco	Roemer 2012
THP	**Tobacco heating product (THP):** Glo and Kent Neosticks (Bright Tobacco) British American Tobacco a. Tobacco b. Heat source c. Mouthpieces d. Electronics and battery e. Device body 	245	Heating of a tobacco substrate	Forster 2017 (this series)
THS	**Tobacco heating product (THP):** iQOS and Marlboro Heatsticks (Essence), Philip Morris International a. Tobacco b. Heat source c. Mouthpieces d. Electronics and battery e. Device body 	340	Heating of a tobacco substrate	Forster 2017 (this series) Schaller 2016

Fig. 1. The three test articles compared in this study: a 3R4F reference cigarette; and two THPs, THP1.0 (Eaton et al., 2017) and THS (Schaller et al., 2016; Smith et al., 2016).

Fig. 2. The Borgwaldt RM20S Smoking Machine for *in vitro* testing. Key features include the eight independent dilution syringes, which enable a single dose–response to be generated in one run (seven dilutions plus one laboratory air sham at the highest dilution used in the respective run), and bespoke switch rig (dual linear arrangement of eight next-generation product mouthpieces), allowing the traditional cigarette carousel to be bypassed for THP testing. The THP1.0 device is shown on the switch rig (circle pop out). The THS device was tested on the same rig. Each syringe was connected to an *in vitro* exposure chamber delivering diluted aerosol to H292 bronchial epithelial cells at the ALI (not shown).

arrangement of 8 mouthpieces) attached to the RM20S (Fig. 2) bypassed the rotating cigarette carousel and enabled a set of THP devices to be held in place and heat activated while another set were being puffed. Activation and heating of devices were synchronised with the 30 s interval puffing regime. A modified HCI regime (HCI_m) was employed for both THPs in which the consumable filter vents were not blocked (because this affects aerosol temperature particularly for THP1.0 (Forster et al., 2017), but otherwise the 55 ml, 2 s, 30 s puffing parameters were unchanged. Seven biologically relevant aerosol dilutions were generated using the RM20S, ranging from 1:20 to 1:10,000 (aerosol:air, v:v) for the reference cigarette and 1:2 to 1:200 for both THPs (Table 2). The dilution range selection for the cigarette was chosen to allow a dose–response curve to be generated from viability to complete cytotoxicity (0% viability), as previously demonstrated by Azzopardi et al. (2015); the THP dilution range was selected based on dose–response data previously obtained for an e-cigarette (Azzopardi et al., 2016), in the knowledge that the cell system would need a higher concentration of aerosol to obtain a cellular response after 1 h exposure. Each dilution was independently tested 4 times for 3R4F and THS ($n = 4$) as established products with supporting historic/published datasets; for THP1.0, as a newly designed product, an extra three repeats were added to verify the accuracy of the findings ($n = 7$). The negative controls were: a 'sham' control (cells exposed to laboratory air for 1 h at the same puffing regime at the highest dilution used in the respective run); a submerged incubator control and ALI incubator control. The positive control consisted of cells that were exposed to 1 ml of basal and 0.5 ml of apical sodium dodecyl sulphate at 350 μM.

2.2. Quality control of exposure system: syringe precision assessment by hydrocarbon analysis

The robustness of the Borgwaldt RM20S was assured by assessing and calibrating dilution syringe precision and accuracy. Tolerance limits were set within 10%, deeming it fit for purpose as

Table 1
Puff-by-puff nicotine (mg/puff), mean puff nicotine (mg/puff) and mean stick nicotine (mg/stick) measured at the source of aerosol generation on the Borgwaldt RM20S: 3R4F Kentucky reference cigarette 10 puffs at HCI, THP1.0 8 puffs at HCI_m, and THS12 puffs at HCI_m (n = 3/product).

3R4F (mg)	Puff	1	2	3	4	5	6	7	8	9	10	11	12	Puff mean	Stick mean	Stick target
	Mean	0.094	0.159	0.157	0.171	0.230	0.251	0.256	0.253	0.211	0.209	–	–	0.199	1.992	1.64–2.45
	SD	0.025	0.026	0.016	0.012	0.033	0.017	0.012	0.025	0.011	0.006	–	–	0.054	0.053	(Eldridge et al., 2015)
THP1.0 (mg)	Puff	1	2	3	4	5	6	7	8	9	10	11	12	Puff mean	Stick mean	Stick target
	Mean	0.023	0.095	0.084	0.052	0.077	0.068	0.047	0.025	–	–	–	–	0.059	0.472	0.43–0.50
	SD	0.04	0.011	0.015	0.008	0.010	0.007	0.006	0.004	–	–	–	–	0.027	0.027	(Forster et al., 2017)
THS (mg)	Puff	1	2	3	4	5	6	7	8	9	10	11	12	Puff mean	Stick mean	Stick target
	Mean	0.068	0.089	0.100	0.118	0.119	0.124	0.124	0.125	0.121	0.109	0.100	0.098	0.108	1.294	1.16–1.48
	SD	0.024	0.030	0.008	0.014	0.013	0.016	0.005	0.010	0.008	0.008	0.013	0.010	0.021	0.018	(Schaller et al., 2016)

Table 2
Nicotine concentration (ng/ml) in cell media after 1 h cellular exposure. Seven aerosol dilutions were generated by using the Borgwaldt RM20S, ranging from 1:20 to 1:10,000 (aerosol:air, v:v) for the reference cigarette and from 1:2 to 1:200 (the same 7 dilutions) for both THPs. Sham refers to the air control (n = 4 exposures for 3R4F and THS, n = 7 for THP1.0).

3R4F (ng/ml)	Dilution	1:2	1:5	1:10	1:20	1:40	1:60	1:100	1:200	1:400	1:1000	1:10,000	SHAM
	Mean	–	–	–	7863	4755	–	2248	1353	3023	545	413	84
	SD	–	–	–	672	721	–	351	41	147	352	260	69
THP1.0 (ng/ml)	Dilution	1:2	1:5	1:10	1:20	1:40	1:60	1:100	1:200	1:400	1:1000	1:10,000	SHAM
	Mean	15,050	9408	5788	–	2535	1613	1174	1161	–	–	–	131
	SD	2387	2745	604	–	517	375	319	362	–	–	–	24
THS (ng/ml)	Dilution	1:2	2	1:10	1:20	1:40	1:60	1:100	1:200	1:400	1:1000	1:10,000	SHAM
	Mean	28,150	17,200	10,153	–	4443	2823	2208	2095	–	–	–	165
	SD	3594	2430	1693	–	110	218	754	943	–	–	–	71

previously described (Adamson et al., 2011).

Eight RM20S dilution syringes (A–H) were calibrated by using a methane standard gas. This quality control measure is critical prior to product testing on the smoking machine to ensure aerosol dilution accuracy and repeatability of delivery within set tolerance limits across all syringes used. It also gives greater confidence in the biological response obtained. The syringe precision and accuracy testing approach and hydrocarbon analysis method are described in detail in Adamson et al. (2011) and Kaur et al. (2010). In brief, a methane test gas (10% CH_4 in nitrogen, Air Products PLC, Crewe, Cheshire, UK) was supplied to each syringe and diluted at a calibration ratio of 1:193 (test gas: air, v:v) by puffing at a known regime for 3 min. The diluted test gas was collected in empty Douglas bags and quantified by using a hydrocarbon analyser (3010 MINIFID portable heated flame ionisation detector total hydrocarbon analyser, Signal Instruments, Willow Grove, PA, USA).

2.3. Puff-by-puff assessment of product aerosol generation using nicotine quantification

Generation of aerosol at source on the RM20S was assessed by quantifying nicotine in each puff from the three test products. The 3R4F reference cigarette was smoked for 10 puffs at the HCI regime; THP1.0 was smoked for 8 puffs at the HCI_m regime; and THS was smoked for 12 puffs at the HCIm regime (Table 1). Each puff was captured in line on a clean 44 mm diameter Cambridge filter pad, and nicotine was quantified by ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), as described in Adamson et al. (2016). Each product was assessed in three independent replicate experiments (n = 3).

2.4. Cell culture

Cell culture was conducted as previously described in Azzopardi et al. (2015). In brief, NCI-H292 human bronchial epithelial cells (American Type Culture Collection, Teddington, Middlesex, UK) were cultured in supplemented Roswell Park Memorial Institute (RPMI) 1640 medium (10% foetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin) at 37 °C, 5% CO_2 in a humidified atmosphere. Forty-eight hours before exposure, cells were seeded at a density of 2×10^5 cells/ml in 0.5 ml of supplemented RPMI 1640 medium apically on 12 mm porous Transwell culture inserts, in sterile 12-well plates, supported with 1 ml of supplemented RPMI 1640 medium in the basal compartment of each well. The apical and basal culture media were replaced with UltraCULTURE™ media (Lonza, Basel, Switzerland) supplemented with 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin 24 h before exposure.

2.5. Cell-viability assessment by Neutral Red Uptake (NRU)

The NRU protocol was based on guidelines set out by the National Institute of Environmental Health Sciences ((National Institutes of Health, 2001)), and was carried out as described in Azzopardi et al. (2015). In brief, after exposure, cells and inserts were washed twice with phosphate buffered saline (PBS). Neutral Red dye (0.05 g/L in UltraCULTURE™) was added apically and basally to the culture inserts, which were incubated for 3 h at 37 °C, 5% CO_2 in a humidified atmosphere. Cells were washed twice with PBS to remove unincorporated dye. Incorporated Neutral Red was eluted from cells by incubation with 500 μl of de-stain solution (50% ethanol, 49% distilled water, 1% glacial acetic acid; v:v:v) and shaken for 10 min at 300 rpm. Aliquots of the NRU eluates (100 μl) were read on a microplate spectrophotometer at 540 nm using a reference filter of 630 nm. Background measurements from blank culture inserts were subtracted from the untreated and treated cells. NRU levels of treated cells were expressed as a percentage of air-exposed controls.

2.6. Nicotine quantification in cell culture media

Quantifying nicotine in cell culture media after THP and reference cigarette exposure enabled an assessment of the amount of aerosol delivered to the cell cultures. Post aerosol exposure, 1 ml of

the medium was removed from the exposure chamber, 10 μl of d_4-nicotine standard was added to the sample and, after sample concentration, the samples were resuspended in solvent and analysed by UPLC-MS/MS as described in Adamson et al. (2016).

2.7. Data analysis

All raw data were processed in Excel 2016 (Microsoft, Redmond, WA, USA). The syringe precision and accuracy assessment plot (Fig. 3) was produced in Minitab 17 (Coventry, UK), with 10% tolerance limits set by the Supplier Quality Requirements Taskforce (2002). The exposed media dosimetry boxplot (Fig. 4) was produced in Minitab. Cell viability assessments (Figs. 5 and 6) were produced in Prism version 7.01 (GraphPad, San Diego, CA, USA). Statistical software SAS version 9.4 (SAS Institute, Cary, NC, USA) was used to perform the statistical analysis and compare the biological response slopes of three products on a dilution basis (Fig. 5) and a nicotine exposure basis (Fig. 6). Products were assessed via a two-step approach as described by Scott et al. (2013), whereby $p < 0.01$ was considered significant. We assume that there is linearity when the quadratic term of the linear regression is not significant. This approach is extremely sensitive to small deviations and, therefore, we increased the significant threshold to 0.01. This is supported by regression being robust to small deviations from linearity, so even for those cases when the p-value of the quadratic term could be between 0.01 and 0.05 the statistical analysis is still reliable for interpretation. First, regression of product and dilution (log-transformed) were fitted to the linear portion of the responses to assess whether their slopes crossed (F-test of interaction). Second, if slopes were found to be statistically different at $\alpha = 0.01$, concentration-to-concentration comparisons between THPs were performed by using linear functions with the LSESTIMATE statement$\alpha = 0.05$. LSESTIMATE statement is a procedure in SAS software that allows calculation of Least Square means which are preferable for carrying out comparisons between unbalanced groups.

3. Results

3.1. Syringe assessment

The Borgwaldt RM20S syringes were calibrated for dilution precision and accuracy, and were monitored weekly to ensure that dilutions were maintained during machine usage and cleaning (after each product was tested). Syringe dilution was assessed at the calibration dilution of 1:193 (test gas:air). At a dilution of 1:193, a target of 520 ppm methane would be expected after bag collection post-syringe. Tolerance limits of 5% and 10% of the target value were employed, with the expectation that less than 10% variability in the data would still deem the tool (the dilution syringes) fit for purpose (Supplier Quality Requirements Taskforce, 2002). Syringe calibration data were obtained over the 8 weeks of the study and were shown to be within expected tolerance limits throughout testing (Fig. 3). All syringe measurements taken were within 10% tolerance (Fig. 3). These data demonstrated that the RM20S generated precise aerosol dilutions and that all syringes were fit for purpose for our *in vitro* product testing.

3.2. Nicotine assessment of aerosol at source generation and in the cell culture media after exposure

Aerosol generation using the RM20S was assessed and confirmed to be in line with analytically published values for each product. Puff-by-puff nicotine concentration was quantified, and nicotine profiles were observed for all three products (Table 1). The reference 3R4F cigarette gave a typical profile; the mean nicotine

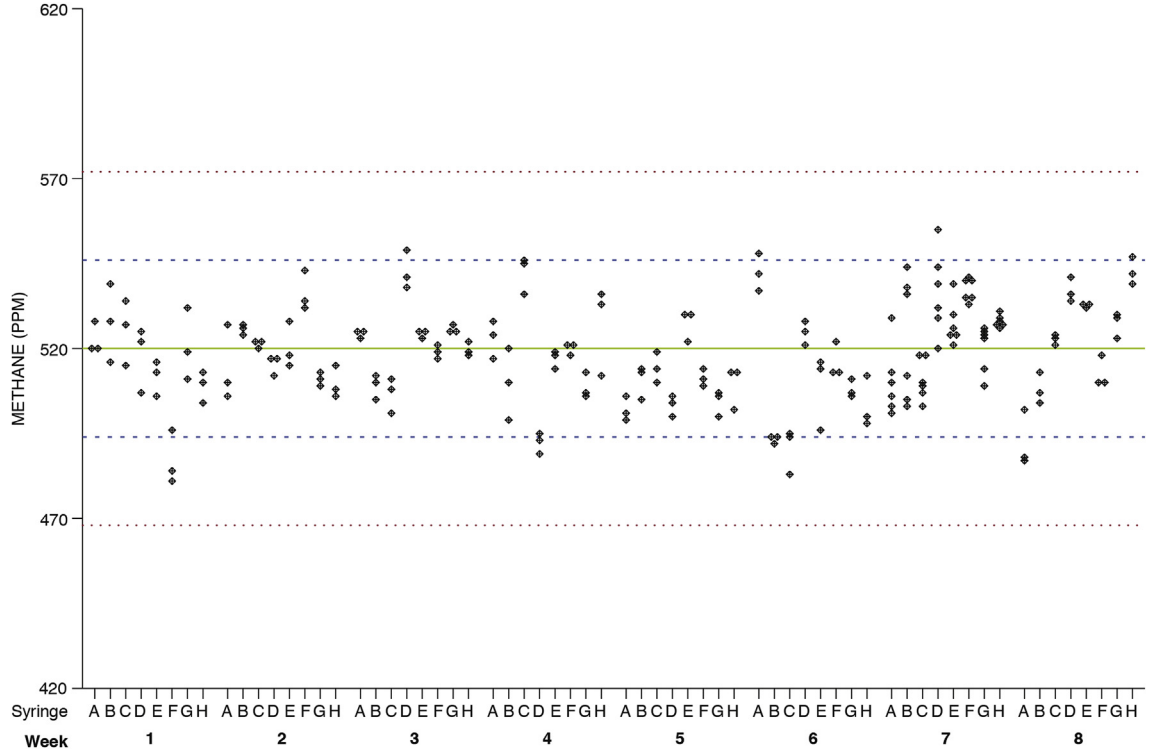


Fig. 3. Individual value plot showing Borgwaldt RM20S syringe dilution calibration, precision and accuracy measurements over the 8-week period of this study. Three independent syringe measurements were taken per syringe per week. At a calibration dilution of 1:193 (test gas: air), the target value is 520 ppm (solid green line). Tolerances were set at 5% (494 ppm and 546 ppm; dashed blue lines) and 10% (the fit-for-purpose threshold, 468 ppm and 572 ppm; dotted red line).

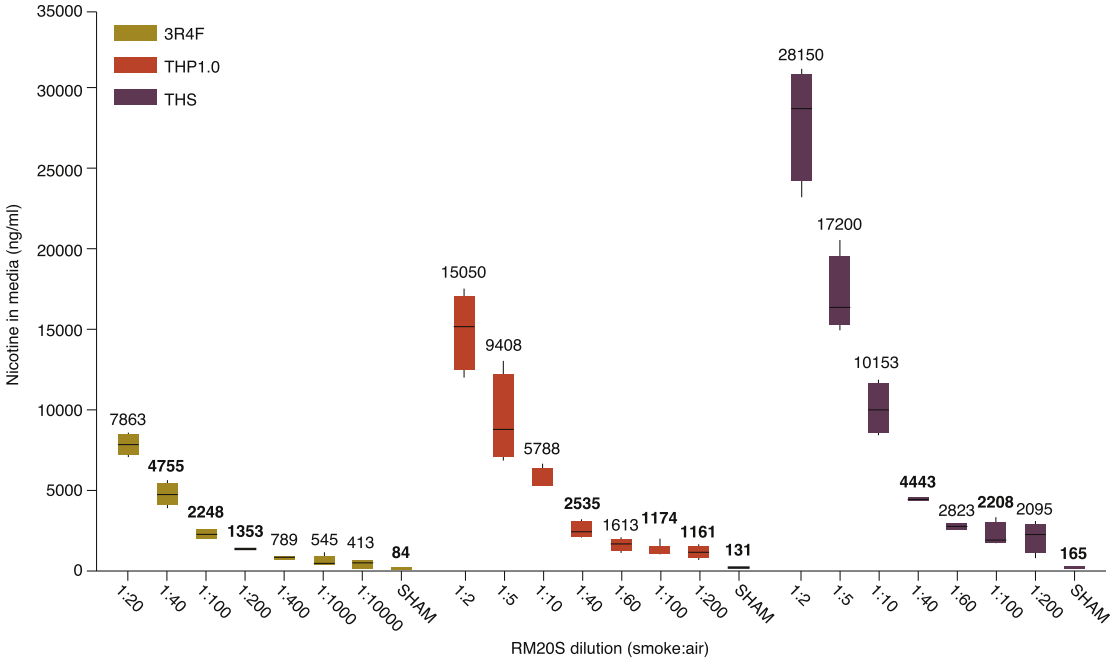


Fig. 4. Boxplot showing cell media nicotine concentration after 1 h cellular exposure. Seven aerosol dilutions were generated by using the Borgwaldt RM20S, ranging from 1:20 to 1:10,000 (aerosol:air, v:v) for the reference cigarette and from 1:2 to 1:200 (the same 7 dilutions) for both THPs. The mean values are displayed above each bar and common dilutions between the cigarette and the two THPs are shown in bold. Sham refers to the air control (n = 4 exposures for 3R4F and THS, n = 7 for THP1.0).

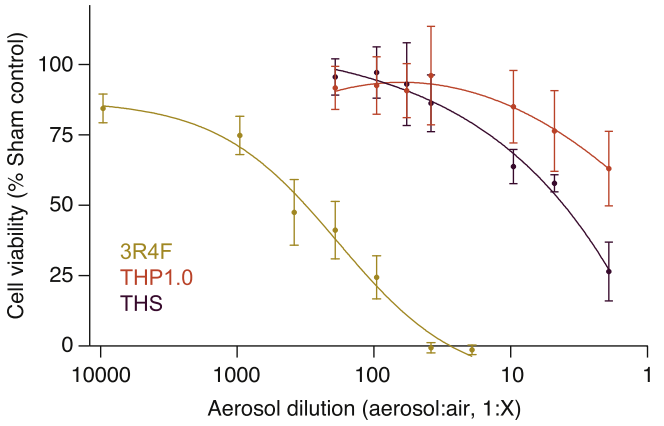


Fig. 5. Neutral Red Uptake (NRU)-determined cell viability of NCI-H292 bronchial epithelial cells after 1 h exposure to a range of dilutions of the three test articles, generated on the Borgwaldt RM20S. Seven aerosol dilutions were generated, ranging from 1:20 to 1:10,000 (aerosol:air, v:v) for the 3R4F reference cigarette, and from 1:2 to 1:200 for both THPs (n = 4 exposures per dilution for 3R4F and THS, n = 7 exposures per dilution for THP1.0). There was a statistical difference in the biological response among all 3 products ($p < 0.0001$) but no difference between the two THPs against aerosol dilution ($p = 0.0152$, where $p < 0.01$ is considered significant). Error bars are standard deviations for replicates at the same dilution, and the type of curve fitting applied to the data is a non-linear regression for the best-fit value of the slope and intercept.

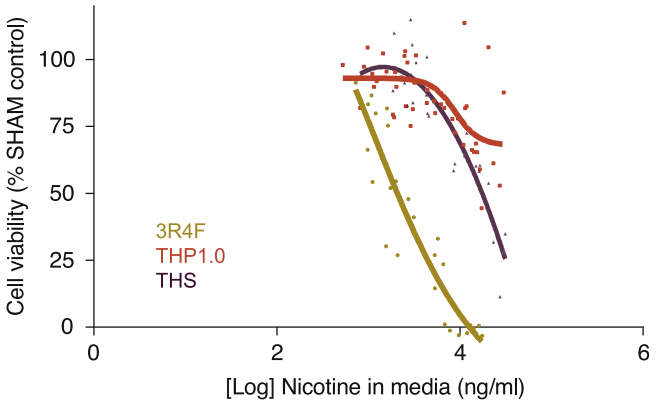


Fig. 6. Neutral Red Uptake (NRU)-determined cell viability of NCI-H292 bronchial epithelial cells after 1 h exposure to a range of dilutions of the three test articles, generated on a Borgwaldt RM20S. Biological response data are presented as a function of nicotine concentration in the exposed media for each independent population of cells. There was a statistical difference in the biological response between the 3 products ($p < 0.0001$) but no difference between the two THPs against nicotine in media ($p = 0.0186$, where $p < 0.01$ is considered significant).

concentration per puff ranged from 0.094 ± 0.025 mg/puff to 0.256 ± 0.012 mg/puff. For the THPs, the mean nicotine concentration per puff ranged from 0.023 ± 0.004 mg/puff to 0.095 ± 0.011 mg/puff for THP1.0, and from 0.068 ± 0.024 mg/puff to 0.125 ± 0.010 mg/puff for THS. The mean puff nicotine concentration (the mean of all puffs per product) was 0.199 ± 0.054 mg/puff for 3R4F, 0.059 ± 0.027 mg/puff for THP1.0, and 0.108 ± 0.021 mg/puff for THS. The mean consumable (stick) nicotine concentration (the sum of all puffs per product) was within acceptable/published range: for 3R4F, it was 1.992 ± 0.053 mg/stick, with a target of 1.64–2.45 mg/stick (Eldridge et al., 2015); for THP 0.1, it was 0.472 ± 0.027 mg/stick, with a target of 0.43–0.50 mg/stick (Forster et al., 2017); and for THS, it was 1.294 ± 0.018 mg/stick, with a target of 1.16–1.48 mg/stick (Schaller et al., 2016).

Nicotine concentration was quantified in the cell culture media after aerosol exposures. There were 7 dilutions plus a sham air control for each product tested. For the cigarette, the dilution ranged from 1:20 to 1:10,000 (aerosol: air, v:v); for both THPs, the same 7 dilutions were selected, ranging from 1:2 to 1:200. For 3R4F cigarette exposures, the cell media nicotine concentration ranged

from 413 ± 260 ng/ml to 7863 ± 672 ng/ml, with an air control value of 84 ± 69 ng/ml (Fig. 4 and Table 2). For THP1.0 and THS, the cell media nicotine concentration ranged from 1161 ± 362 ng/ml to 15,050 ± 2387 ng/ml, with an air control value of 131 ± 24 ng/ml, and for THS from 2095 ± 943 ng/ml to 28,150 ± 3594 ng/ml, with an air control value of 165 ± 71 ng/ml (Fig. 4 and Table 2). The air control nicotine values were acceptable and represented background readings from the exposure and measurement systems (nicotine is always measured in air controls because in previous experiments the molecule permeates tubing and any other plastics that it contacts and then leach out over time; we have observed sham controls at 350 ng/ml, but never observed 0 ng/ml; data not shown).

3.3. Cell viability assessment

After 1 h exposure to a range of aerosol dilutions from three products and a 24 h post-exposure recovery period, cell viability was quantified by NRU assay. For each dilution treatment, viability was expressed as a percentage of the air control (sham). The acceptance criterion for exposed treatments was that the (untreated) incubator controls were within ±20% of the sham; all NRU data shown here fell within this acceptance criterion (data not shown).

For 3R4F reference cigarette-exposed cells, a classic sigmoidal dose–response viability curve was recorded (n = 4): cell viability ranged from 85 ± 5% at the highest dilution of smoke (1:10,000) to −1 ± 2% at the highest concentration of smoke (1:20) (Fig. 5 and Table 3). Because negative viability values were obtained when blanks were subtracted from the NRU extract readings, this in fact indicated 0% viability (100% cytotoxicity).

Both THP products demonstrated a similar shift to the right relative to the 3R4F dose–response curve, indicating that a greater exposure concentration was required to elicit reductions in cell viability. Such a shift demonstrates that the test article is less cytotoxic than with the reference product. For the cells exposed to THP1.0 aerosol, a shorter dose response was observed because even at the highest concentration of aerosol exposure, complete cytotoxicity was not achieved. Cell viability ranged 92 ± 8% at the highest dilution of aerosol (1:200) to 63 ± 13% at the highest concentration of aerosol (1:2) (Fig. 5 and Table 3). The SDs for THP1.0 are noticeably larger than those for THS and 3R4F, despite more measurements being performed for the THP1.0 (n = 7) than for the other two products (n = 4). The THP devices tested have different heating mechanisms resulting in different nicotine profiles per puff. Through puffs 1–12, THS has a relatively consistent profile for nicotine delivery, whereas THP1.0 has a distinct double peak in nicotine delivery throughout the 8 puffs (Table 1).

For the THS-exposed cells, a similar (truncated) dose–response was observed within the same dilution range because, again, complete cytotoxicity was not achieved even at the highest concentration of aerosol exposure (n = 4): cell viability ranged from 96 ± 6% at the highest dilution of aerosol (1:200) to 27 ± 10% at the highest concentration of aerosol (1:2) (Fig. 5 and Table 3).

Cells were exposed for the same duration (1 h), with products generating aerosol at the same puffing regime (HCI or HCIm; however, product delivery per puff was different (Table 1) due to the tobacco blend) and, in the case of the THPs, their heating profiles: THP1.0 heats to a maximum of 240 °C ±5 °C (Eaton et al., 2017) and THS heats to a maximum of 350 °C (Smith et al., 2016). Thus, under the same exposure conditions, each cell population was exposed to the same dilution range for the two THPs, but they would have received different doses (of measured nicotine) at these dilutions.

In an alternative representation of the biological response data, cell viability was presented against exposed nicotine in the cell media for each individual chamber exposure (Fig. 6). The cell viability values obtained above were plotted against exposed media nicotine concentration, with a curve of best fit applied. This approach showed that all three products started in the region of ≥85% viability. The 3R4F reference cigarette demonstrated a linear response, where cell viability decreased as nicotine concentration in the exposed media increased; the biological response from the two THP devices was pulled closely together initially, but diverged slightly as nicotine concentration in the exposed media increased. Statistically there were differences in the biological response slopes between the three products ($p < 0.0001$). When 3R4F was removed from the regression, the p-value of the slopes between the THPs was not significant against dilution ($p = 0.0152$) (Fig. 5) or against nicotine ($p = 0.0186$) (Fig. 6).

4. Discussion

Building upon our current knowledge of *in vitro* exposure systems and NGP aerosols, in this study we assessed aerosol generation and cytotoxicity from two commercially available THPs (THP1.0 and THS) relative to tobacco smoke from 3R4F reference cigarettes. We employed a staged approach to this testing, confirming the nicotine delivery of the tested products across the *in vitro* exposure system and comparing the biological response data from all products.

As quality control, we monitored syringe performance by using hydrocarbon analysis and control charts during aerosol generation to confirm that the Borgwaldt RM20S Smoking Machine was fit for purpose. Hydrocarbon analysis of syringe-diluted methane test gas was assessed over an 8-week period, and was shown to be between 5% and 10% of the calculated target of 520 ppm (Fig. 3). These data are supported by previously published Borgwaldt RM20S syringe studies, where individual syringe precision and accuracy of aerosol dilutions were shown to be fit for purpose (<10%) (Adamson et al., 2011; Kaur et al., 2010).

Aerosol generation was characterised by quantifying the

Table 3
Neutral Red Uptake (NRU)-determined cell viability of H292 lung epithelium after 1 h exposure to a range of dilutions of the three test articles, generated on the Borgwaldt RM20S Smoking Machine. Biological data were calculated as a percentage of the air control; thus, sham was always 100% (n = 4 exposures for 3R4F and THS, n = 7 for THP1.0).

3R4F	Dilution	1:2	1:5	1:10	1:20	1:40	1:60	1:100	1:200	1:400	1:1000	1:10,000	SHAM
(%)	Mean	–	–	–	−1	−1	–	25	41	48	75	85	100
	SD	–	–	–	2	2	–	8	10	12	7	5	0
THP1.0	Dilution	1:2	1:5	1:10	1:20	1:40	1:60	1:100	1:200	1:400	1:1000	1:10,000	SHAM
(%)	Mean	63	77	86	–	97	91	93	92	–	–	–	100
	SD	13	15	13	–	18	11	10	8	–	–	–	0
THS	Dilution	1:2	2	1:10	1:20	1:40	1:60	1:100	1:200	1:400	1:1000	1:10,000	SHAM
(%)	Mean	27	58	64	–	87	94	98	96	–	–	–	100
	SD	10	3	6	–	10	15	9	6	–	–	–	0

concentration of nicotine in each puff at the generation of source aerosol in the RM20S. Each of the three products gave a different and unique puffing profile (Table 1). The ascending puff profile from the cigarette was typical and expected, conforming with previous observations (Adamson et al., 2016; Adamson et al., 2017). THP1.0 demonstrated a repeatable 'M-shaped' profile, where nicotine concentration increased and decreased twice through the puff numbers (1–8), peaking at puff numbers two and five. This profile would be expected based on the heating profile of the device with two heater segments (Fig. 1) that are separately controlled by the inbuilt software (Eaton et al., 2017), driving aerosol generation and thus nicotine concentration each time that the device temperature increases. THS gave a linear nicotine puff profile over the longest duration of puffs (12 per consumable), with a slight and gradual increase in nicotine.

The acceptance criteria for aerosol generation prior to biological testing were set so that the products must generate mean consumable (stick) nicotine yields within published values. Herein we showed the Borgwaldt RM20S generated aerosols within acceptable and expected analytical range (Table 1). 3R4F was 1.992 ± 0.053 mg nicotine/stick, while the target was 1.64–2.45 mg nicotine/stick (Eldridge et al., 2015); THP1.0 was 0.472 ± 0.027 mg nicotine/stick, while the target was 0.43–0.50 mg nicotine/stick (Forster et al., 2017); THS was 1.294 ± 0.018 mg nicotine/stick, while the target was previously established as 1.16–1.48 mg nicotine/stick (Schaller et al., 2016).

In parallel to the cell viability assessment using the NRU assay, nicotine was quantified in the exposed cell culture medium from each chamber by using UPLC-MS/MS (Fig. 4 and Table 2). Nicotine measurement in exposed media benefitted the study two-fold: 1) cellular exposure to THP aerosol was proven in a dose-responsive manner (crucial when presenting data of a reduced biological response, because there is physical evidence of cellular/test article interaction); and 2) it enabled presentation of the biological response not only as arbitrary smoking machine ratio dilutions (not the easiest approach to extrapolate to other exposure systems) but also against a measured exposure metric across products (nicotine), enabling cross-reference with a wider population of disparate laboratory exposure scenarios. Although we do not assume that other components or toxicants within the aerosols follow the same profile of nicotine, it provides an exposure marker common among all products, aligning systems and exposure endpoints.

In addition, dosimetric evaluation is important when testing new nicotine products such as THPs, because predictions of dosing based on the physicochemical properties of the original aerosol can mislead and affect the relevance of *in vitro* studies (Steiner et al., 2016). The cigarette appeared to give the lowest measured concentration of nicotine in the exposed media, but this was due to the higher dilution range selected for 3R4F exposure (1:20–1:10,000 vs. 1:2–1:200) (Fig. 4). Both THP products had the same dilution range (1:2–1:200), but THS delivered more to the exposure media than THP1.0; this is consistent with the respective nicotine content and deliveries per puff and per consumable of the two THPs (Table 1), driven by their different temperature profiles (Eaton et al., 2017; Smith et al., 2016).

Cell viability was determined by NRU assay using an *in vitro* human bronchial epithelial cell culture system (NCI-H292), exposed for 1 h at the ALI; a complete dose response was demonstrated for the 3R4F reference cigarette (Fig. 5 and Table 3). The reference cigarette can also be used as an internal study standard with comparison to historic data from previous studies, to give confidence to the data generated. The same viability profile previously obtained from the 3R4F during an e-cigarette cytotoxicity assessment (Azzopardi et al., 2016) was matched in this THP study (EC_{50} was the same for both data sets, $p = 0.3852$, F-test comparison data not shown). The two THPs were also assessed for cell viability. When compared with 3R4F responses, THPs demonstrated a rightward shift of the dose–response curve relative to 3R4F, indicating that higher THP concentrations were required to elicit reductions in cell viability. However, both THPs gave truncated dose–responses: 0% viability could not be achieved within the same exposure time and dilution range (Fig. 5 and Table 3). This was even the case with THP1.0, which was tested more than the other two products on seven independent occasions. Because this was replicated in all independent experimental runs for both THPs, we can reasonably conclude that this truncation was not a chance observation. A dilution of 1:2 is the most concentrated aerosol that the RM20S Smoking Machine can deliver. Therefore, future studies to increase dose and drive greater cytotoxicity should consider an increase in exposure duration beyond 1 h for the 1:2 dilution. There is also the possibility of utilising exposures with undiluted aerosols to generate a biological response within 1 h. Considering the overlapping RM20S dilutions among the three products, we achieved 0% cell viability with the 3R4F (100% cytotoxicity) at a dilution of 1:40 (aerosol:air), while THP1.0 showed 97% viability and THS showed 87% viability at the same dilution (Fig. 5 and Table 3). At a glance, this suggests that the THPs elicit a slight difference in biological responses. However, the concentration of nicotine in their aerosols also differs (Table 1). By presenting biological response against exposed cell media nicotine concentration (Fig. 6), the responses between the two THPs aligned more closely. When 3R4F was removed from the statistical analyses, there was no significant difference in the observed biological response between the two THPs ($p = 0.0152$ for response against dilution, Fig. 5; and $p = 0.0186$ for response against media nicotine, Fig. 6) (the THP slopes were significant at $p < 0.05$ at the highest concentration only, but were not significant at 0.01). Furthermore, presenting biological response data against exposed nicotine concentration quantified in the exposure media would facilitate future product comparisons at longer exposure durations (or shorter exposure durations with undiluted NGP aerosols), enabling a complete dose response from all products and better statistical comparisons, for example, by EC_{50}.

These biological data (Figs. 5 and 6) demonstrate the reduced cytotoxic potential of THP1.0 and potentially also the THP category, relative to a conventional cigarette. Adding greater confidence to the observations in our study, the same conclusions have been drawn from independent investigations, where *in vitro* toxicological assessment of THP aerosol revealed a >90% reduction in cytotoxicity relative to 3R4F smoke, as determined by NRU assay (Schaller et al., 2016; Smith et al., 2016). Reassuringly, other studies have confirmed similar and significant differences between a reference cigarette and different THPs when tested *in vitro* (Doolittle et al., 1990; Foy et al., 2004; Munakata et al., 2017).

5. Conclusions

As part of a collection of various scientific assessments conducted and presented in this issue, we assessed the cytotoxicity of THP aerosol relative to cigarette smoke. In this study, we compared the *in vitro* cytotoxic response of NCI-H292 human lung epithelial cells to reference cigarette smoke (3R4F) and aerosol from two THPs (THP1.0 and THS). The two THPs demonstrated a statistically similar, substantially reduced biological response as compared with tobacco smoke, as indicated by a shift in the dose–response curve induced by the THPs relative to the viability profile obtained from 3R4F cigarette exposure. A complete viability dose response was observed for 3R4F smoke. For both THPs, complete cytotoxicity was not observed even after a 1 h exposure at the highest concentration of vapour in the exposure system (1:2 aerosol:air). Comparing the

three products at the common dilution of 1:40, when complete loss of viability was observed for 3R4F, the two THP products still showed more than 87% cell viability. The appropriateness of the exposure system used for this assessment was confirmed, with dilution syringes shown to be precise, repeatable and fit for purpose, delivering aerosols from three different test articles within published acceptance criteria. Crucially, and despite a reduced biological response from the THPs, delivery of the test article to the exposure chamber was proven by dosimetric characterisation of cell media nicotine, and shown to be greater from both THPs than from the cigarette: in other words, more concentrated aerosol was delivered but the biological response was less.

The range of emerging NGPs with reduced toxicant emissions offers the potential of reduced-risk tobacco and nicotine products. E-cigarettes and THPs are two such examples, with e-cigarettes delivering nicotine in a liquid droplet aerosol, and THP vapour being generated by heating tobacco at temperatures <350 °C. Preclinical assessment data, such as the *in vitro* dosimetric and cytotoxic assessment described herein, can support a weight of evidence approach to assess the reduced risk potential of these next generation tobacco and nicotine products.

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Transparency document

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Author contributions

Tomasz Jaunky, Jason Adamson, David Thorne, Damien Breheny and Marianna Gaca conceived the study. Tomasz Jaunky and Jason Adamson managed and performed the experimental work and analysed the data. Simone Santopietro and Anya Terry supported the experimental work. Jason Adamson prepared the manuscript. David Thorne, Damien Breheny and Marianna Gaca reviewed the data analysis and draft manuscript. Christopher Proctor oversaw the research programme. All authors read and approved the final manuscript.

Declaration of interest

The authors declare that there are no conflicts of interest. All authors are employed by British American Tobacco.

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