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# Assessment of novel tobacco heating product THP1.0. Part 6: A comparative *in vitro* study using contemporary screening approaches

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ABSTRACT

Cigarette smoking is a major risk factor for many adverse health conditions. Novel tobacco heating products (THPs) heat tobacco, reducing exposure to many of the harmful combustion toxicants in conventional cigarette emissions. *In vitro* studies have been employed to support the toxicological evaluation of chemicals and complex mixtures, including cigarette smoke. The use of automated robotics platforms for *in vitro* toxicological screening complements traditional testing approaches. Multiparametric toxicity and oxidative stress endpoints were used to assess *in vitro* biological responses elicited after exposure to total particulate matter (TPM) from two commercially available THPs, and the reference tobacco product 3R4F, in human bronchial epithelial cells. A luciferase-based reporter gene assay was used to assess antioxidant response element (ARE) transcriptional activation in stably transfected H292 cells after 6 and 24 h exposures. High-content screening was used to assess 10 endpoints normal human bronchial epithelial cells after 4 or 24 h exposures. 3R4F TPM stimulated significant increases in ARE activation ($p < 0.005$) and moderate activity in HCS cell-based assays compared to THP at comparable doses. THPs showed little or no activity in all assays. HCS techniques can extend safety assessments providing information quickly in the early stages of product innovation and development.



## 1. Introduction

In the past decade, there has been significant progression in the development, design and application of *in vitro* test systems. The technological advancement of automated robotic analysis and detection instrumentation, paired with the accessibility and stability of *in vitro* cellular cultures, have further enabled these tools. Ongoing innovations in cellular and molecular biology have facilitated a paradigm shift in toxicology testing, away from the traditional heavy reliance on low-throughput animal data towards the greater use of medium- and high-throughput *in vitro* cellular screening technologies. The National Research Council's publication, "Toxicity Testing in the 21st Century: A Vision and a Strategy" (NRC, 2007), outlined approaches using advances in molecular biology, biotechnology and *in vitro* and computational sciences to help evaluate the health risks of consumer products and safety assessment of chemicals. The US Environmental Protection Agency's ToxCast safer chemicals research programme has developed and is applying novel toxicological approaches to evaluate chemicals for potential health effects. These efforts have contributed to the Toxicology in the 21st Century Federal Agency consortium, bringing together the Environmental Protection Agency, National Toxicology Program at the National Institute of Environmental Health Science, the National Institutes of Health's National Center for Advancing Translational Sciences and the Food and Drug Administration. Robotic technology is used to screen tens of thousands of chemicals and is helping to drive a change in toxicological assessment.

These medium- and high-throughput screening approaches therefore, may act as a precursor to *in vitro* studies that precede classical toxicological evaluations. Such screening approaches

*Abbreviations:* ARE, antioxidant response element; DMSO, dimethyl sulphoxide; HCI, Health Canada intense smoking regimen; $HCI_m$, modified Health Canada Intense smoking regime; HCS, high content screening; ISO, International Standards Organization; NHBE, normal human bronchial epithelial cells; c-Jun, c-Jun stress kinase; p-H2AX, phosphorylated H2Ax histone; ROS, reactive oxygen species; TPM, total particulate matter; THP1.0, Tobacco Heating Product version 1; THS, Tobacco Heating System; 3R4F, University of Kentucky reference cigarette.

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harness cell lines and cultures to monitor changes in markers or proteins associated with biological activity that might indicate potential toxic effects. Using immunohistochemistry approaches, these markers can be located across different subcellular components, including the cytoplasm, cell membranes, mitochondria and the nucleus, and their distribution may be assessed. High-content screening (HCS) enables the parallel assessment of multiple endpoints. Fluorescent tags are used to label markers in live cells, and by using automated image analysis, such as flow cytometry or microscopy, marker or protein expression and the percentage of cells expressing the target marker can be detected in real time. Using fluorophores with different absorption and emission spectra facilitates the simultaneous measurement of several markers at a time, with the maximum reported so far being 17 (Nierode et al., 2016).

Non-invasive techniques, in particular, reporter gene assays (RGA), can improve understanding of intracellular responses and provide greater granularity than immunohistochemistry. RGA can be used for cell-based screening, to study gene expression at the transcriptional level or the activity of a specific promoter. Most reporter genes incorporate fluorescent or luminescent proteins that are stimulated when the target promoter initiates transcription and the reporter gene is expressed. Reporter cell lines have been constructed for specific gene expression evaluation, including oxidative stress and inflammation (Sekine et al., 2016; Taylor et al., 2016) and can be easily automated for HCS approaches. The use of HCS platforms, such as image analysis and RGA, have been applied widely for the rapid screening of drug candidates in the early stages of drug discovery (Nierode et al., 2016) and used to evaluate diverse compound libraries consisting of many chemicals and nanomaterials (Collins et al., 2017). Most recently, they have been used for screening the chemical and biological safety of product innovations, including tobacco and nicotine next-generation products (NGPs) (Iskandar et al., 2016; Marescotti et al., 2016).

Cigarette smoking is a major risk factor for many adverse health conditions, including cardiovascular disease, respiratory disease and lung cancer (US DHHS, 2014). Recently, the use of novel NGPs, including electronic cigarettes (e-cigarettes) and tobacco heating products (THPs) (Bombick et al., 1997; Branton et al., 2011; Dittrich et al., 2014; Doolittle et al., 1990; Goniewicz et al., 2014; Margham et al., 2016; Schaller et al., 2016; Smith et al., 2016). These devices work on the premise of avoiding conventional combustion processes that are the source of many toxicants found in cigarette smoke (Eaton et al., 2017; Foy et al., 2004; Poynton et al., 2017; Schaller et al., 2016). The use of such products may reduce the adverse health effects compared to the use of traditional tobacco products (Institute of Medicine, 2011); (Stratton et al., 2001).

THPs are still relatively new to the consumer, but awareness is growing rapidly, as is the consumer acceptability of these products as a viable alternative to cigarette smoking. THP technologies employ different methods of heating tobacco, with some of the more recent devices designed to control the heating profile (Eaton et al., 2017; Ishikawa et al., 2016; R.J. Reynolds Tobacco Company, 1988; Smith et al., 2016). Overall however, these products liberate fewer toxicants that conventional cigarettes (Eaton et al., 2017; Schaller et al., 2016) and have demonstrated lower biological activity in laboratory based *in vitro* toxicological and biological tests (Doolittle et al., 1990; Foy et al., 2004; Jaunky et al., 2017; Thorne et al., 2017).

In this study, contemporary screening approaches were used to assess multiparametric toxicity and oxidative stress endpoints for *in vitro* biological responses to total particulate matter (TPM) from two commercially available THPs, THP1.0 and a tobacco heating system (THS) and the 3R4F reference cigarette in human bronchial epithelial cells (NCI-H292) or normal human bronchial epithelial cells (NHBE). Utilising a luciferase-based reporter assay, the transcriptional activation of the antioxidant response elements (ARE) in stably transfected NCI-H292 cells was assessed following 6 h and 24 h exposure to 3R4F and THP TPMs. With a HCS approach employing the ArrayScan VTI (ThermoFisher Scientific, Dartford, UK) platform, 10 different toxicity and oxidative stress end points, including ATP, cell count, glutathione content, mitochondrial mass, mitochondrial membrane potential, nuclear size, formation of reactive oxygen species (ROS), DNA structure, DNA damage and the c-Jun stress kinase, were measured in NHBEs following 4 h or 24 h exposures to 3R4F or THP TPMs. The assays selected in this study were based on *in vitro* endpoints that have been previously applied to assessment of tobacco smoke toxicants, THPs or NGPs (Gonzalez-Suarez et al., 2016; Iskandar et al., 2016; Kogel et al., 2015; Marescotti et al., 2016; Taylor et al., 2016).

## 2. Materials and methods

### 2.1. Chemicals and reagents

Chemicals were sourced from Sigma Aldrich (Haverhill, UK) unless otherwise stated. The H292-ARE-Luc2P reporter cells were designed and constructed by Promega Corporation (Madison, WI, USA). The ONE-Glo Luciferase Assay substrate was purchased from Promega (Southampton, UK).

### 2.2. Products

Three products were used in this study: a scientific reference cigarette, 3R4F (University of Kentucky, Lexington, KY USA) and two THPs. Tobacco heating product, version 1 (THP1.0) and tobacco heating system (THS) are both commercially available in Japan. The composition, construction and mainstream smoke chemistry yields from the 3R4F have been previously reported by Roemer et al. (2012). Product specifications are detailed in Eaton et al. (2017) for THP1.0 and Schaller et al. (2016) for THS. Specifications for all test products are detailed in Table 1.

### 2.3. TPM generation

Reference 3R4F cigarettes were conditioned as per the International Organization for Standardization (ISO) guideline 3402:1999 (ISO, 1999) and smoked on a Borgwaldt RM200A machine (Borgwaldt-KC, Hamburg, Germany). Tobacco consumables for THP1.0 and THS were also conditioned in accordance with ISO 3402:1999. THPs were puffed on a Borgwaldt LM20X (Borgwaldt-KC) linear machine. Up to 150 mg of TPM was collected onto a 44 mm Cambridge filter pad (Whatman, Maidstone, UK). The pads were weighed before and after smoking to determine the mass of the deposited TPM. Pads were extracted into dimethyl sulphoxide (DMSO) to a final stock concentration of 24 mg/ml of TPM. The extracts were stored in single-use aliquots at −80 °C. TPMs were analysed for glycerol, nicotine, water, and nicotine free dry particulate matter (NFDPM), using gas chromatography thermal conductivity (GC-TCD) and flame ionisation detectors (GC-FID). Glycerol was measured for 3R4F, THP1.0 and THS at 2.3 ± 0.1, 2.7 ± 0.2 and 3.6 ± 0.3 mg/cig respectively; nicotine was analysed as 1.9 ± 0.1, 0.4 ± 0.02, 1.1 ± 0.04 mg/cig respectively; water was 12.0 ± 0.5, 8.5 ± 0.6, 15.9 ± 1.0 mg/cig respectively and NFDPM was 24.3 ± 0.8, 6.0 ± 0.7, 10.0 ± 0.6 mg/cig respectively.

### 2.4. H292-ARE-Luc2P reporter cell culture

Human bronchial epithelial cells NCI-H292 (American Type Culture Collection) were transfected with a pGL4[ARE-luc2P/Hygro] vector to generate a luciferase-based reporter cell line, as

**Table 1**
Overview of product specifications.

| Parameter | Product | | |
|---|---|---|---|
| | 3R4F reference cigarette[a] | THP1.0[b] | THS[c] |
| Product | Scientific 3R4F research reference cigarette | Commercial THP | Commercial THP |
| Smoking regimen for TPM generation | Health Canada intense[d] | Modified Health Canada intense (no vent blocking) | Modified Health Canada intense (no vent blocking) |
| Smoking profile | Bell | Bell | Bell |

Abbreviations: THP = tobacco heating product; THS = tobacco heating system.
[a] Roemer et al., 2012.
[b] Eaton et al., 2017.
[c] Smith et al., 2016.
[d] Health Canada Official Method T-115 (puff volume 55 ml, puff interval 30 s, puff duration 2 s, 100% vent blocking).

previously described (Taylor et al., 2016). H292-ARE-Luc2P reporter cells were maintained in RPMI-1640 medium, with sodium bicarbonate, supplemented with 10% foetal bovine serum (GE Healthcare Life Sciences, Hatfield, Hertfordshire, UK), 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and hygromycin B (150 μg/ml) and maintained at 37 °C in a humidified 5% carbon dioxide ($CO_2$) incubator. Ninety-six well plates were seeded with 10,000 cells per well that were allowed to adhere and grow to confluency over 72 h at 37 °C with 5% $CO_2$ prior to experimental exposures.

### 2.5. Viability assessments and activation of antioxidant response elements (ARE)

H292-ARE-Luc2P cell viability was measured within the 96-well plates following exposure for 6 h or 24 h, using the CellTiter-Glo assay (Promega) as per manufacturer's protocol and as reported previously (Taylor et al., 2016). Briefly, cells were lysed with CellTiter-Glo Assay reagent (Promega) to enable the generation of a luminescent signal that is proportional to the cellular ATP concentration and directly proportional to the cell number. Relative luminescence units were recorded with a 1 s integration time on a SpectraMax Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). These units are expressed as a percentage of the control compared to treatment conditions, up to 200 μg/ml TPM.

Transcriptional activation of the ARE was determined following either a 6 h or 24 h exposure of the stably transfected H292-ARE-Luc2P cells to TPM from the three products at concentrations from 0 to 200 μg/ml. Luminescence signals were measured on a SpectraMax Multi-Mode Microplate Reader with a 1 s integration time, directly after the addition of the ONE-Glo reporter substrate. Positive controls, 3 mM potassium bromate or 30 μM D, L-sulforaphane, were used to confirm activation of the ARE in the H292-ARE-Luc2P reporter cell assay.

ARE RGA results were analysed with a general linear model (ANOVA) to identify statistically significant differences in mean assay responses between products. Dunnett's multiple comparison tests were performed to identify significant assay responses to individual test product TPM concentrations. Values of $p < 0.05$ were considered to be statistically significant. Data are mean relative luminescence units of six replicate wells repeated over four independent experiments. All assay responses shown were normalised to the vehicle control (DMSO 0.83%) and expressed as fold change in assay signal. Statistical outliers (identified in Minitab) were omitted prior to statistical analysis.

### 2.6. HCS by fluorescence cell imaging

The experimental protocol was conducted as previously described (Gonzalez-Suarez et al., 2016; Kogel et al., 2015; Marescotti et al., 2016). Briefly, black, clear-bottomed 96-well cell culture plates were initially seeded with 10,000 NHBE cells per well followed by a 24 h incubation at 37 °C. Three separate wells containing NHBE cells were then exposed to a concentration range (0–120 μg/ml, 0.5% DMSO) of the test product or assay controls for 4 h or 24 h. Following treatment, NHBE cells were stained with the specific dye or antibody for each endpoint. Cell imaging with fluorescence analysis was performed on a Cellomics ArrayScan VTI High Content Screening platform and vHCS software (ThermoFisher Scientific). A minimum of 20 individual image acquisitions were taken for each well of the experimental plates.

Raw fluorescence intensity values (RFU) were normalised to the (0.5% DMSO) vehicle control in all cases and expressed as fold change in assay signal. The response was considered significant if the fold change was more than 1.5 for ROS formation, nuclear size, c-Jun stress kinase or DNA damage (phosphorylation of H2AX) assays or values by less than 30% in the case of ATP, cell count, glutathione content, mitochondrial mass or mitochondrial membrane potential assay endpoints (Table 2). Raw vales are included in Supplementary Data.

### 2.7. Statistics and data analysis

All analyses were conducted in line with the experimental approach, as discussed under each section. Data are presented for ARE as fold change over control (0.83% DMSO) or for HCS endpoints, as a ratio of control (0.5% DMSO).

## 3. Results

### 3.1. Viability assessments and activation of ARE

The CellTiter-Glo viability test was performed following 6 h or 24 h exposure of H292-ARE-Luc2P reporter cells to the test product TPMs (0–200 μg/ml). 3R4F TPM was found to induce up to 25% cytotoxicity following 24 h exposures at 166 and 200 μg/ml, with no decreases in cell viability after 6 h exposures. Neither THP decreased cell viability during either 6 h or 24 h exposures across all dilutions tested. Table 3 shows the viability data obtained from the Cell Titer-Glo assay for all products, time points and concentrations tested.

The pattern of responses in the H292-ARE-Luc2P RGA following a 6 h or 24 h exposure to TPM are shown in Fig. 1. Activation of the ARE promoter-linked luciferase reporter was induced following exposures to TPM from all test products ($p < 0.005$). Similarly, activation of H292-ARE-Luc2P cell-line reporter function was measured, following stimulation with the Nrf2 activator D, L-sulforaphane (30 μM) or the pro-oxidant, potassium bromate (3 mM).

3R4F TPM exposure at either 6 h or 24 h induced a significantly

**Table 2**
HCS assay end points overview.

| HCS endpoint | Probe | Molecular target | Cellular implication or cellular event | Assay control |
|---|---|---|---|---|
| ATP | CellTiter-Glo | Respiratory chain | Inhibition of metabolism measured via a decrease in ATP production | Rotenone and L-buthionine-sulfoximine |
| Cell count | Hoechst 33342 or Syto11 | Cell number | Antiproliferative, apoptotic or necrotic effects measured through cell counts | |
| Glutathione content | Monochlorobimane | GSH | Measurement of GSH levels; decrease indicates loss due to the presence of ROS or covalent binding, increase may result from protective cellular responses to oxidative stress | |
| Mitochondrial mass | MitoTracker Deep Red | Total mitochondrial mass | Measurement of mitochondrial mass post-exposure indicates potential effects due to oxidative stress and associated damage | |
| Mitochondrial membrane potential | MitoTracker Deep Red | $\Delta\psi m$<br>ATP production | Decreased mitochondrial membrane potential indicates an impaired cellular energy production, potentially resulting in mitochondrial toxicity and apoptosis. | |
| Nuclear size | Hoechst 33342, Syto11 | Nuclear area | Increase in nuclear area indicates necrosis or cell-cycle arrest; decrease can indicate apoptosis | |
| ROS formation | Dihydroethidium | Intracellular ROS generation | Increased ROS generation indicates the formation of toxic superoxide intermediates resulting from oxidative stress | |
| DNA structure | Hoechst 33342, Syto11 | DNA fragmentation | Increased DNA structure indicates fragmentation of DNA or chromosomal instability | |
| DNA damage | Anti p-H2AX antibody | Phosphorylation of the histone H2AX at Ser139 | Increased DNA damage (p-H2AX) indicates the presence of double-strand breaks. | Mitomycin C |
| Stress kinase activation | Anti-c-Jun antibody | Phosphorylation of c-Jun | Increased phosphorylation of c-Jun indicates activation of the stress kinase pathway that activate numerous protective responses | Colchicine |

Abbreviations: HCS = high-content screening; GSH-glutathione sulfhydryl; ROS = reactive oxygen species; p-H2AX = phosphorylated H2AX.

**Table 3**
Viability data using Cell Titer-Glo.

| Exposure time (h) | Product | Total particulate matter dose (μg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 7.4 | 22 | 66 | 100 | 166 | 200 |
| 6 | 3R4F | 100.00 ± 3.21 | 99.74 ± 5.65 | 99.33 ± 5.51 | 96.47 ± 6.51 | 97.99 ± 5.89 | 94.89 ± 6.93 | 93.27 ± 9.07 |
| | THP1.0 | 100.00 ± 4.55 | 100.21 ± 4.85 | 101.69 ± 4.78 | 104.00 ± 7.99 | 99.08 ± 6.58 | 101.57 ± 4.35 | 96.61 ± 6.54 |
| | THS | 100.00 ± 4.07 | 100.29 ± 4.96 | 99.47 ± 5.91 | 105.71 ± 12.07 | 100.19 ± 3.82 | 100.00 ± 3.91 | 96.73 ± 6.62 |
| 24 | 3R4F | 100.00 ± 2.60 | 99.58 ± 4.71 | 99.23 ± 4.86 | 92.88 ± 6.11 | 85.42 ± 3.31 | 77.90 ± 2.12 | 75.70 ± 4.67 |
| | THP1.0 | 100.00 ± 2.68 | 100.78 ± 6.26 | 99.74 ± 7.99 | 102.15 ± 3.47 | 103.06 ± 4.46 | 100.87 ± 4.76 | 101.98 ± 4.25 |
| | THS | 100.00 ± 2.00 | 100.35 ± 4.15 | 100.88 ± 5.23 | 101.33 ± 5.19 | 102.00 ± 5.79 | 101.88 ± 4.14 | 101.39 ± 5.24 |

Data are means ± standard deviations.

($p < 0.005$) greater activation of the ARE than either the THP1.0 or THS products across all doses tested. TPM for both THPs caused little activation of the ARE RGA and when compared to each other, and were not significantly different after either 6 h or 24 h exposure ($p > 0.05$). Fig. 1 demonstrates the observed responses to all products following exposures (Fig. 1a and b) and the responses from the THP products only (Fig. 1c and d). Supplementary Table 1 shows the RLU data obtained from the ARE assay for all products, time points and concentrations tested.

Exposure to low concentrations of 3R4F TPM induced a concentration-dependant ARE activation which plateaued at 66 μg/ml eliciting a 9 and > 30-fold increase in activity at 6 and 24 h exposures respectively. This was followed by a return to near baseline in reporter activity with exposures to 3R4F TPM concentrations above 66 μg/ml following 6 h exposure and maintaining up a 20-fold increase in reporter activity at 24 h. A plateau is observed at 100 μg/ml in the 24 h treatment, with a decrease in activity at 166 μg/ml. This bell-shaped response in the absence of significant cytotoxicity may demonstrate negative feedback cell signalling responses, indicating a potential overwhelming of the cellular antioxidant system. A very slight (3-and 2-fold) increase in reporter activity was observed in a concentration-dependent manner following exposure to THP TPMs, at 6 and 24 h exposures respectively.

### 3.2. HCS assessments

The effects of TPM from 3R4F and THPs were further investigated using multiparametric HCS, with a total of 10 end points assessed over serial dilutions at 4 and 24 h exposures. A summary of the results are shown in Table 4 and in Figs. 3 and 4, raw vales are included in Supplementary Data. Referring to Figs. 3 and 4, exposure of NHBE cells to TPM from the reference cigarette 3R4F for 4 h, induced moderate reductions in cellular availability of the antioxidant glutathione, ROS generation and ATP, an impaired mitochondrial membrane potential, decrease in cellular nuclear size and DNA structure, in combination with an increased incidence of DNA damage. These responses were modest and most occurred at the higher TPM doses (80–120 μg/ml). These responses indicate initial cellular oxidative stress responses most likely resulting from the generation of superoxide intermediates that may deplete or oxidise glutathione and cause oxidative damage to mitochondria or disrupt the electron transfer chain. By contrast, exposure to the TPM generated from either of the commercially available THPs did not induce a positive signal in any of the HCS end points assessed in this study (Fig. 2).

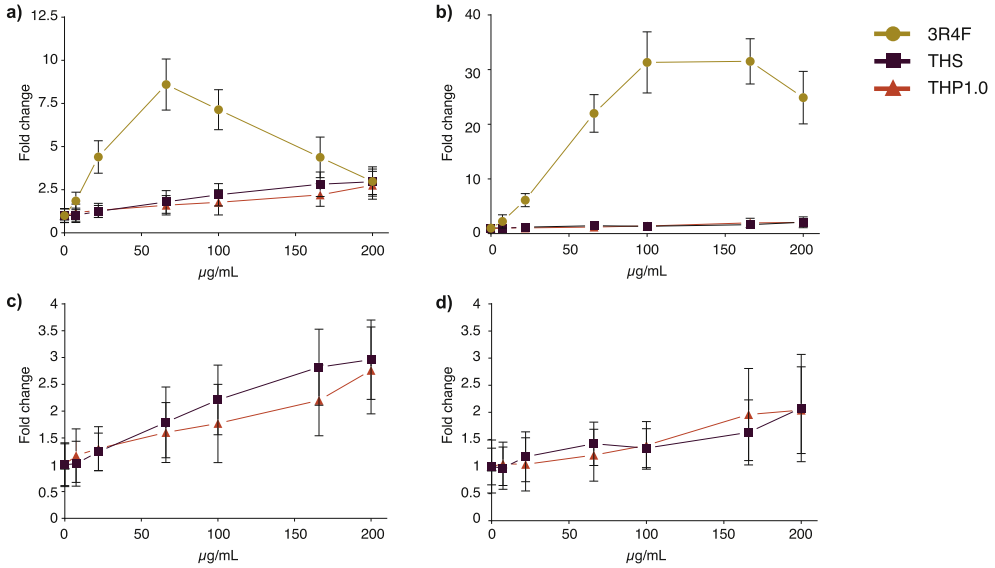


**Fig. 1. Activation of the H292-ARE-Luc2P RGA following 6 and 24h exposure to 3R4F, THP1.0 or THS**. Data shown are mean fold changes in response normalised to the vehicle control (0.83% DMSO). **(a)** 6 h exposure to 3R4F, THP1.0 and THS. **(b)** 24 h exposure to 3R4F, THP1.0 and THS. **(c)** 6 h exposure toTHP1.0 and THS. **(d)** 24 h exposure, to THP1.0 and THS. **(c)** and **(d)** represent same data on a decreased scale to better observe responses.

**Table 4**
HCS results.

| HCS endpoint | Exposure time (h) | 3R4F | THP1.0 | THS |
|---|---|---|---|---|
| ATP | 4 | 60[a] | – | – |
| | 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 | 4 | – | – | – |
| | 24 | 60[a] | – | – |
| Stress kinase | 4 | – | – | – |
| | 24 | – | – | – |

Values are the minimum required TPM concentration (μg/ml) to elicit >1.5-fold increase in assay signal from the 0.5% DMSO vehicle control or <30% decrease in signal for the ATP, cell count, glutathione content, mitochondrial mass and mitochondrial membrane potential assay end points.
[a] TPM-concentration-dependant response.

Following a longer 24 h exposure to 3R4F TPM, small but dose-dependent increases in glutathione content were measured at lower doses (<60 μg/ml) that were mirrored by increased ROS generation, mitochondrial membrane potential and phosphorylation of c-Jun stress kinase and H2AX, indicating an adaptive oxidative stress response of the cell to the treatment. This protective mechanism may involve the upregulation of genes downstream of the ARE promoter, which falls under the control of the ROS-sensitive transcription factor Nrf2. Many of these genes are involved in redox homeostasis and are implicated in glutathione regulation. At higher doses of TPM (>60 μg/ml), cell responses decreased, which could indicate the onset of cellular apoptosis, as indicated by decreased ATP and nuclear size. The responses again observed were moderate, with no higher than a 1.5-fold increase or 30% decrease in response from untreated control (Figs. 3 and 4). Table 4 summaries the results HCS endpoint results.

## 4. Discussion

This study assessed the *in vitro* biological responses to TPM from two commercially available THPs (THP1.0 and THS), and the 3R4F reference cigarette using contemporary screening approaches in NCI-H292 cells or NHBEs. TPM test matrices for all products were generated in the same manner, and equivalent doses were selected for all products as part of a comparative study design. The transcriptional activation of the antioxidant response element (ARE) using a luciferase-based reporter gene assay in H292-ARE-Luc2P reporter cells, as previously described (Taylor et al., 2016), was assessed following 6 h and 24 h exposure to 3R4F and THPs. HCS, which has previously been reported as a viable tool for tobacco product assessment (Gonzalez-Suarez et al., 2016; Kogel et al., 2015; Marescotti et al., 2016), was employed to measure biological processes ranging from cell health and oxidative stress markers to ROS formation and DNA damage in NHBEs following 4 h or 24 h exposures to 3R4F or THP TPMs.

The H292-ARE-Luc2P RGA was shown to be sensitive enough to detect and measure ARE transcriptional activation following exposure to TPMs from both 3R4F, THP1.0 and THS after 6 h and 24 h exposures. 3R4F TPM treatment, however, induced a significantly greater activation of ARE than either of the THP products. 3R4F TPM induced a concentration-dependant ARE activation that peaked at 66 μg/ml eliciting increases in activity of nine-fold and >30-fold after 6 h and 24 h exposures, respectively. ARE activity returned to near baseline as dose increased in 6 h treated cells but maintained activity with higher doses at 24 h exposures. This pattern of response in the absence of significant cytotoxicity may demonstrate a negative feedback cell-signalling response, potentially indicating an overwhelming of the antioxidant system. Wardyn et al. (2015) have previously described a negative feedback system that promotes pro-inflammatory or apoptotic responses to potentially unresolvable oxidative stress, thereby preventing genetic mutations via oxidative DNA damage.

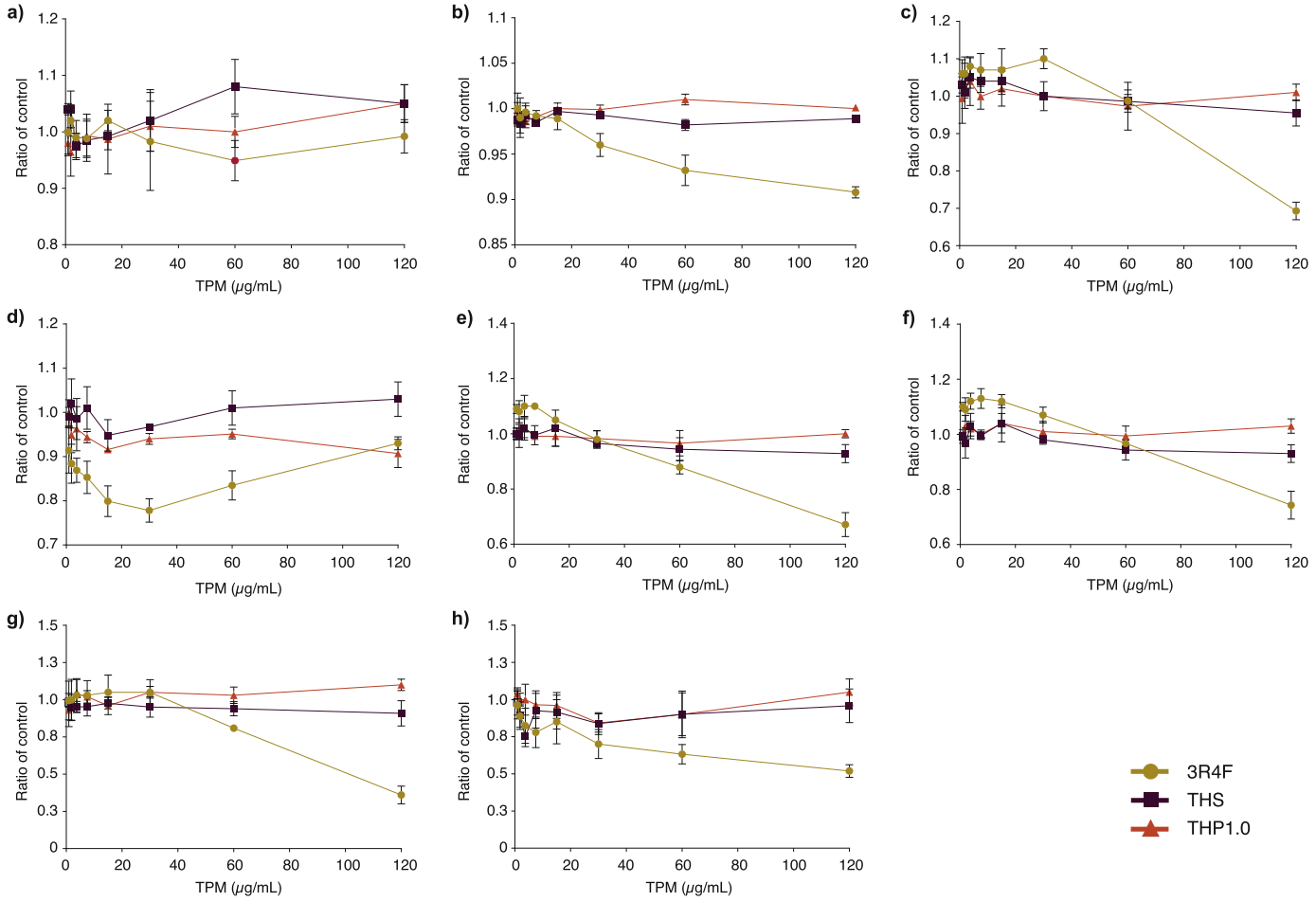


**Fig. 2.** High-content screening for cell health end points after 4 h exposure to 3R4F, THP1.0 or THS. (a) Cell count, (b) nuclear size, (c) DNA structure, (d) mitochondrial mass, (e) mitochondrial membrane potential, (f) formation of reactive oxygen species, (g) glutathione content and (h) cellular ATP. Control was 0.5% dimethyl sulphoxide. Error bars indicate standard deviations.

TPM from both THPs also showed an increase in ARE activation from baseline. However, the responses observed were significantly lower than that of 3R4F, with the maximum fold changes being approximately two-fold and three-fold after 6 h and 24 h exposures, respectively. Furthermore, no significant difference in ARE activation was observed between the two THPs using this RGA.

The study of the activation of the ARE response element in stably transfected H292 cells has not been previously investigated using TPM test matrices from tobacco products, including THPs. The analysis of ARE activation has been previously reported after exposure to aqueous extracts obtained with 10 × 55 ml puffs captured in 20 ml media for 3R4F and e-cigarette aerosols (Taylor et al., 2016). The 3R4F aqueous extracts were associated with activation of the ARE in a dose-responsive manner, but two commercially available e-cigarettes did not induce ARE activity above control levels.

This study also investigated a HCS approach using fluorescence cell-based imaging techniques across several cellular oxidative stress end points. Moderate decreases in ATP, glutathione content, ROS generation, mitochondrial membrane potential, cellular nuclear size and DNA structure were observed in NHBE cells following 4 h treatment with 3R4F TPM. After 24 h exposure to 3R4F TPM, a small but dose-dependent increase in glutathione content was measured at lower doses (<60 μg/ml) mirrored by an increase in ROS generation, mitochondrial membrane potential and phosphorylation of c-Jun stress kinase. This indicates an adaptive oxidative stress response of the cell to the treatment. This protective mechanism may involve the upregulation of genes downstream of the ARE promoter, which falls under the control of the ROS-sensitive transcription factor Nrf2. Many of these genes are involved in redox homeostasis and are implicated in glutathione regulation. At higher doses of TPM (>60 μg/ml) NHBEs decreased their responses, which could indicate the onset of cellular apoptosis, as indicated by a decrease in ATP and nuclear size. The responses again observed were moderate, with no more than a 1.5-fold increase or a 30% decrease in response from untreated control.

3R4F TPM exposure at 4 h and 24 h induced phosphorylation of H2AX, which is an indicator of DNA damage, as has been previously reported (Gonzalez-Suarez et al., 2016; Marescotti et al., 2016). It is important to note, that the dosing did not significantly reduce cell numbers, which indicates little or no cytotoxic effects of 3R4F TPM exposure at the doses tested.

In contrast, exposure to the TPM generated from either of the commercially available THPs did not induce activity or significant responses in the HCS end points assessed in this study, at either timepoint. The lack of biological responses from both THPs reported in this study may be attributed to the reduction in measurable chemical species and toxicants in the aerosol from the THPs compared to 3R4F smoke. THPs typically heat tobacco to temperatures of 250–350 °C, which are significantly lower than those reached during the combustion of tobacco in cigarettes (~900 °C), thus avoiding many of the harmful toxicant emissions

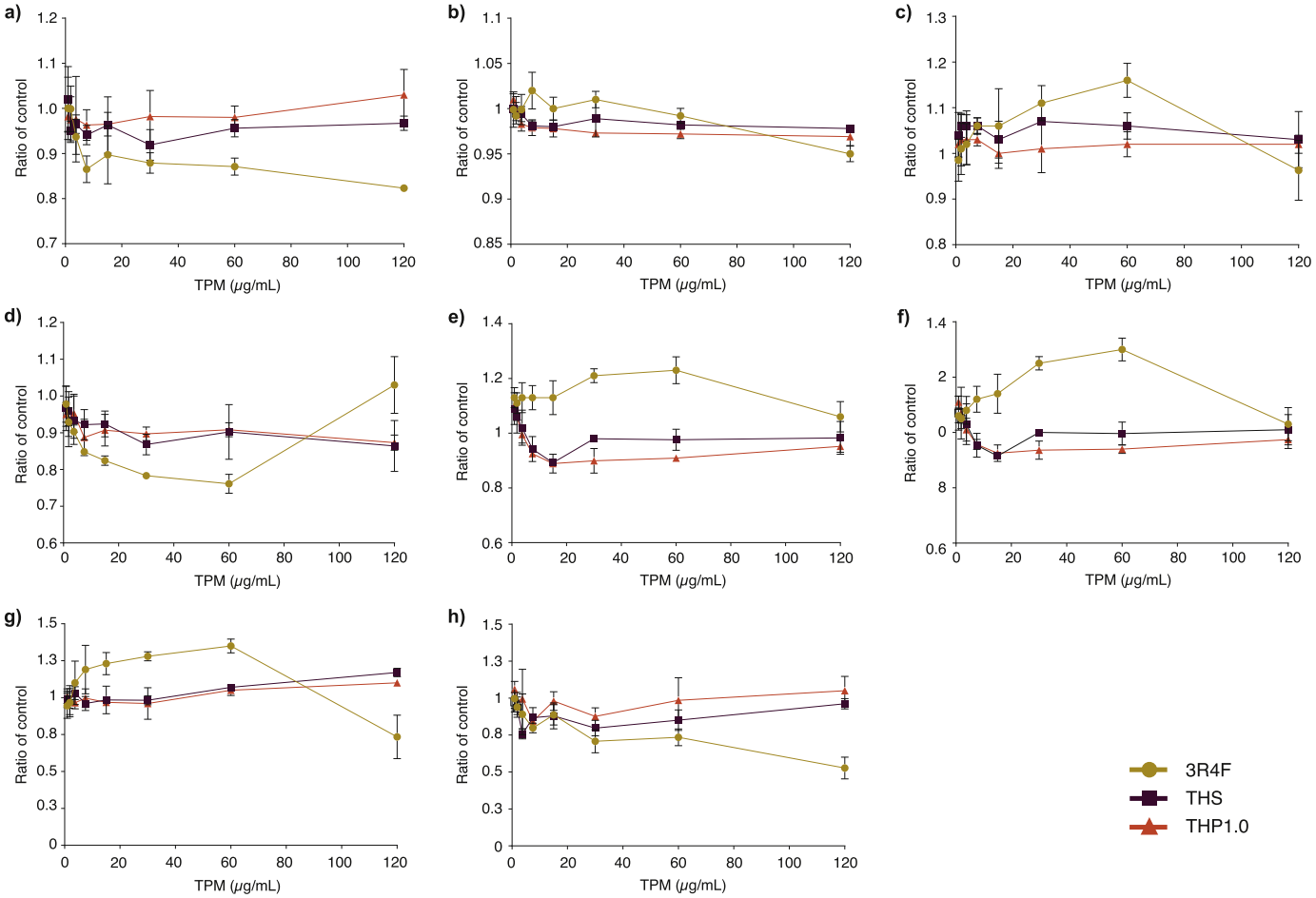


**Fig. 3.** High-content screening for cell health end points following 24 h exposure to 3R4F, THP1.0 or THS TPM. (a) Cell count, (b) nuclear size, (c) DNA structure, (d) mitochondrial mass, (e) mitochondrial membrane potential, (f) formation of reactive oxygen species, (g) glutathione content and (h) cellular ATP. Control was 0.5% dimethyl sulphoxide. Error bars indicate standard deviations.

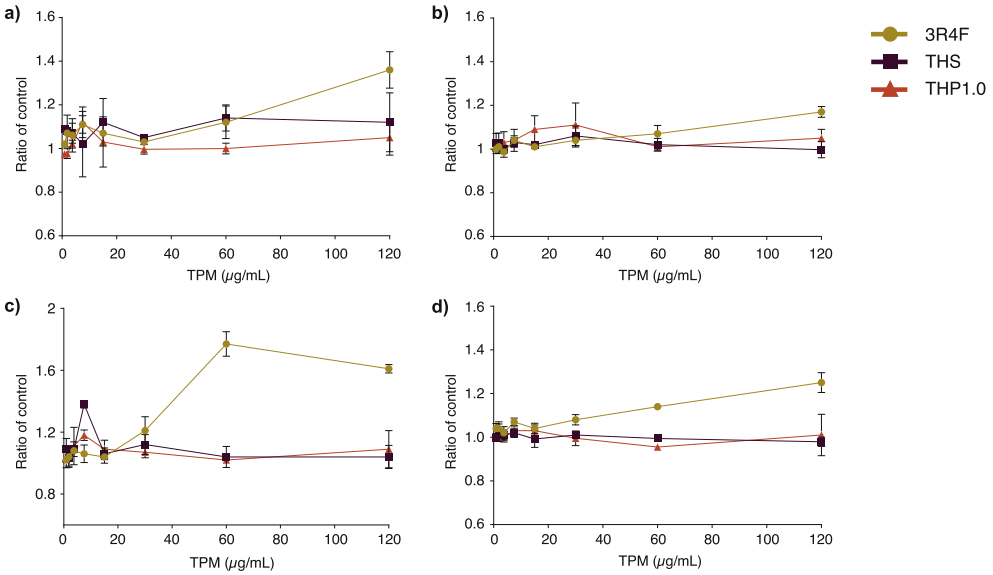


**Fig. 4.** High-content screening for DNA damage and c-Jun stress kinase activation following 4 h and 24 h exposures to 3R4F, THP1.0 or THS TPM. End points were phosphorylation of H2AX after 4 h (a) and 24 h (c) and phosphorylation of c-Jun stress kinase after 4 h (b) and 24 h (d). Control was 0.5% dimethyl sulphoxide. Error bars indicate standard deviations.

(Eaton et al., 2017; Forster et al., 2017; Schaller et al., 2016). Studies have reported significantly reduced levels of harmful and potentially harmful constituents in THPs compared to 3R4F (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; Jaunky et al., 2017; Schaller et al., 2016; Thorne et al., 2017), which support our findings.

In this study TPM test matrices were generated in the same manner, and equivalent doses were selected for all products to form a comparative study design. Future studies will need to investigate higher doses of THP test article to try to drive responses across end points. Inclusion of additional positive controls, such as known tobacco toxicants, as opposed to those used within the assays and end points will ensure the results are appropriately contextualised.

## 5. Conclusions

Application of screening approaches enables the testing of many products, at different concentrations and exposure durations across many endpoints and different cell lines. This study investigated the suitability of contemporary approaches, such as RGAs and fluorescence cell imaging HCS as screening methods to support product assessment for THPs versus 3R4F. TPM from 3R4F tobacco products stimulated responses in the ARE RGA and multiple cellular acute response endpoints that could be assessed with an HCS approach and could show relatively little or no activity with two THP TPMs at comparable doses.

Future studies will investigate increasing TPM exposure concentrations and other possible test matrices, such as aqueous aerosol extracts. The application of repeated dosing or the generation of more concentrated TPM extracts might be sufficient to elicit a concentration-dependent response to help differentiate between NGPs, such as the THPs assessed in this study. The use of HCS techniques may offer opportunities for the safety assessment of tobacco and nicotine NGPs, providing information faster in the early stages of product innovation and development, supporting traditional *in vitro* toxicological approaches.

## Declaration of interest

The authors are employees of British American Tobacco. All experimental work was funded by British American Tobacco. Cyprotex, Nether Alderly, Cheshire, UK, conducted all HCS experimental work. ARE experimental work was carried out at British American Tobacco, Southampton, UK.

## Authors contributions

Mark Taylor and Tony Carr conducted all ARE experimental work and data analysis. Paul Walker managed testing at Cyprotex, UK. David Thorne and Marianna Gaça designed the studies. Christopher Proctor oversaw the research programme. Mark Taylor, Tony Carr, David Thorne, Damien Breheny, Paul Walker and Marianna Gaca contributed to drafting the manuscript and all authors approved the final version.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.yrtph.2017.08.016.

## Transparency document

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