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Assessment of novel tobacco heating product THP1.0. Part 7: Comparative *in vitro* toxicological evaluation

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ABSTRACT

In vitro studies have been widely used to support the toxicological evaluation of chemicals and complex mixtures including cigarette smoke. In this study, the total particulate matter and whole aerosol from a Kentucky reference 3R4F cigarette and two commercially available tobacco heating products (THPs) were assessed using *in vitro* mutagenicity, cytotoxicity and tumour-promoting activity assays. The Ames assay assessed mutagenicity using *Salmonella typhimurium* tester strains TA98, TA100, TA1535, TA1537 and TA102 ± metabolic activation (S9). The mouse lymphoma assay was used with short 3 h and longer 24 h exposures. The Bhas 42 cell transformation assay was incorporated as an *in vitro* alternative for detecting tumour promoters, and the neutral red uptake cell viability assay provided an acute measure of cytotoxicity. To complement the approach, the Ames assay was also employed with *S. typhimurium* tester strains TA98, TA100, TA1535, TA97 and TA102 using a scaled down methodology for the assessment of aerosols. All the *in vitro* techniques employed produced a clear positive response with cigarette smoke and in contrast, a negative response to THPs at doses equivalent to or higher than a cigarette smoke test matrix. The data show little difference between the THPs assessed suggesting parity between products.

1. Introduction

Cigarette smoking is a major risk factor for many adverse health conditions, including cardiovascular disease, respiratory disease and lung cancer (Stratton et al., 2001; US DHHS, 2014). Given these effects, the tobacco industry has spent many years investigating reduced exposure technologies, cigarettes and devices to limit toxicant exposure in those that continue to smoke Institute of Medicine (2011). The chemical composition of smoke from any product or device results from the choice of tobacco blend, the design and/or format and the presence or absence of a filter and filter components, such as charcoal and/or other selective adsorptive materials. Recent examples of technologies aimed at reducing toxicant profiles include substitute tobacco sheet, which acts as a tobacco diluent (McAdam et al., 2001); the development and refinement of cigarette design, format and selective filtration (Bombick et al., 1997; Branton et al., 2011; Dittrich et al., 2014); treatment of tobacco prior to cigarette manufacturing (Liu et al., 2011); agronomic practices (Lewis et al., 2008); and the development of alternative products, such as electronic cigarettes (e-cigarettes) and heat not burn devices (Doolittle et al., 1990; Foy et al., 2004; Goniewicz et al., 2014; R. J. Reynolds Tobacco Company, 1988; Schaller et al., 2016; Smith et al., 1996, 2016).

Next-generation tobacco and nicotine products (NGPs), such as tobacco heating products (THPs) and e-cigarettes, have evolved significantly over recent years and are reaching consumer acceptability. THPs operate by heating a tobacco rod to temperatures up to approximately 350 °C, which is significantly lower than that found during the combustion of cigarettes (>900 °C). At these lower temperatures, the aerosol generated has a less-complex chemical composition compared to that of a conventional cigarette (Eaton et al., 2017; Forster et al., 2017; Schaller et al., 2016). Recent studies have demonstrated that THPs had significantly reduced levels of harmful constituents when compared to a 3R4F reference cigarette (Forster et al., 2017; R. J. Reynolds Tobacco Company 1988; Smith et al., 2016) and have demonstrated reduced toxicity in laboratory-based *in vitro* tests (Breheny et al., 2014; Schaller et al., 2016). Given the development of NGPs, there is a requirement to assess these emerging products to understand how they compare to conventional tobacco products.

As part of an assessment strategy investigating the reduced exposure potential of NGPs, non-clinical testing, including chemical analysis and *in vitro* toxicity testing, can be employed initially as

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Abbreviations

Bhas	Bhas 42 cell transformation assay
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulphoxide
GEF	global evaluation factor
HCI	Health Canada Intense
HCIm	Health Canada Intense modified
MEM	Modified Eagles medium
MLA	Mouse Lymphoma Assay
OECD	Organization for Economic Cooperation and Development
S9	rat liver metabolic activation system
TPA	12-O-tetradecanoylphorbol-13-acetate
TPM	total particulate matter
THP	Tobacco Heating Product
THP1.0	Tobacco Heating Product version 1
THS	Tobacco Heating System
3R4F	University of Kentucky reference cigarette

part of a stewardship approach (Liu et al., 2011; Murphy et al., 2017). As a complement to chemical analysis of emissions (Eaton et al., 2017; Forster et al., 2017, Schaller et al., 2016), a battery of *in vitro* toxicity tests may be used for initial screening of the mutagenic and cytotoxic potential of NGPs (Murphy et al., 2017; Schaller et al., 2016). International guidelines have been developed that recommend an appropriate battery of *in vitro* mutagenicity and carcinogenicity assays to ensure consistency of testing procedures and appropriate assay selection as part of a risk assessment process. Several guidelines exist, including those developed by the International Conference on Harmonisation (ICH 2011), the Committee on Mutagenicity (COM 2011), Health Canada (Health Canada, 2005) and the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA, 2004). In summary, these guidelines recommend the use of i) a bacterial mutagenicity assay (Ames reverse mutation assay), (Maron and Ames, 1983); ii) a mammalian cell based assay for cytogenetics/mutation (*in vitro* micronucleus assay) (Parry et al., 2002); iii) chromosome aberrations or the mouse lymphoma assay (MLA), (Hozier et al., 1981)]; and iv) a cytotoxicity-based assay. The Bhas 42 cell transformation assay is often used to supplement testing approaches and add to a weight of evidence approach for *in vitro* carcinogenicity testing. Although the Bhas 42 cell transformation assay is not a recognised assay for use in regulatory testing, a guidance document has been issued by the Organization for Economic Cooperation and Development (OECD) (OECD, 2016). This assay is considered to add value in its ability to detect non-genotoxic carcinogens and to support a weight of evidence based testing strategy.

The NRU, Ames and MLA assays are used routinely to assess total particulate matter (TPM) from cigarette smoke, and have been extensively employed (Andreoli et al., 2003). The responses from cigarette smoke TPM in these assays have been ubiquitously positive using reference cigarette smoke test articles (Combes et al., 2013; Crooks et al., 2013; Scott et al., 2013). Additionally, these assays have been used in an assessment strategy to compare traditional combustible cigarettes and have shown distinguishing potential (Combes et al., 2012). Furthermore, these techniques and approaches have been used to assess the genotoxic and cytotoxic potential of NGPs including e-cigarettes and THPs (Azzopardi et al., 2016; Foy et al., 2004; Schaller et al., 2016; Thorne et al., 2016). The results from these assays have shown that these products are either negative or extremely low-responding compared to traditional tobacco smoke, which appears to correlate with the reduction in levels of chemical compounds and toxicants found in the source aerosol compared to tobacco smoke.

In this study, TPM from a Kentucky reference cigarette (3R4F) was compared to TPM generated from two THPs, commercially available in Japan: Tobacco Heating Product version 1 (THP1.0) and Tobacco Heating System (THS). The NRU assay was employed for assessment of acute cytotoxicity; the Ames assay as a measure of bacterial mutagenicity, the MLA as a mammalian mutagenicity test and supplemented with the Bhas 42 cell transformation assay. To further complement these studies, whole aerosols were assessed from the three products using the Ames assay and a scaled-down 35 mm agar plate methodology.

2. Materials and methods

2.1. Chemicals and reagents

Where specified, Aroclor-1254-induced rat liver post-mitochondrial supernatant (S9) mix (Moltox™, Boone, NC, USA) provided metabolic activation. All other chemicals were obtained from Sigma-Aldrich (Dorset, UK), unless otherwise stated.

2.2. Products

Three products were assessed in this study; a scientific reference cigarette (3R4F) and two commercially available tobacco heating products: THP1.0 and THS sourced from Japan. An overview of specifications for the three products assessed in this study are provided in Table 1, and more technical product details on consumables and emission chemistry data are detailed for the THP1.0 in Eaton et al. (2017) and for THS in Schaller et al. (2016).

2.3. Total particulate matter (TPM) generation

TPM was generated in a comparable manner for each product. Reference 3R4F cigarettes and THP consumables were conditioned as per International Organization for Standardization (ISO) guideline 3402:1999 (ISO, 1999) and puffed, respectively, on a Borgwaldt RM200A2 and a Borgwaldt LM20X linear machine (Borgwaldt-KC, Hamburg, Germany). The Health Canada Intense (HCI) smoking regime (55 ml puff volume, 2 s puff duration and 30 s puff interval, 100% vent blocking; Health Canada Official Method T-115 (HCI 1999)) was used for 3R4F, and a modified HCI regime modified (HCI_m) without vent blocking, which is not possible for THPs. Up to 150 mg TPM was collected onto 44 mm Cambridge filter pads (Whatman, Maidstone, UK) that were weighed before and after smoking to determine the mass of the deposited material. Pads were extracted into dimethyl sulphoxide (DMSO) to a final stock concentration of 24 mg/ml. TPMs were stored in single use aliquots at −80 °C.

For all products, 'partner' pads were smoked/puffed on each day of TPM generation. In addition, for every 10 3R4F pads collected, a quality control (QC) pad was also collected under the ISO condition. Partner and QC pads were sent to the BioPharmaceutical CMC Solutions – small molecules Department (Covance, Harrogate, UK) for analysis of nicotine, water and glycerol content by GC-TCG and GC-FID.

A summary of the test article TPM characterisation can be found in Table 2.

2.4. Whole aerosol (WA) exposure

Two Vitrocell VC 10 smoking robots (Vitrocell systems, Waldkirch, Germany), serial numbers VC10/090610 and VC10/060614,

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