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A novel hybrid tobacco product that delivers a tobacco flavour note with vapour aerosol (Part 2): *In vitro* biological assessment and comparison with different tobacco-heating products

Damien Breheny*, Jason Adamson, David Azzopardi, Andrew Baxter, Emma Bishop, Tony Carr, Ian Crooks, Katherine Hewitt, Tomasz Jaunky, Sophie Larard, Frazer Lowe, Oluwatobiloba Oke, Mark Taylor, Simone Santopietro, David Thorne, Benjamin Zainuddin, Marianna Gaça, Chuan Liu, James Murphy, Christopher Proctor

Research and Development, British American Tobacco Investments Ltd, Regents Park Road, Southampton, Hampshire SO15 8TL, UK

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ABSTRACT

This study assessed the toxicological and biological responses of aerosols from a novel hybrid tobacco product. Toxicological responses from the hybrid tobacco product were compared to those from a commercially available Tobacco Heating Product (c-THP), a prototype THP (p-THP) and a 3R4F reference cigarette, using *in vitro* test methods which were outlined as part of a framework to substantiate the risk reduction potential of novel tobacco and nicotine products. Exposure matrices used included total particulate matter (TPM), whole aerosol (WA), and aqueous aerosol extracts (AqE) obtained after machine-puffing the test products under the Health Canada Intense smoking regime. Levels of carbonyls and nicotine in these matrices were measured to understand the aerosol dosimetry of the products. The hybrid tobacco product tested negative across the *in vitro* assays including mutagenicity, genotoxicity, cytotoxicity, tumour promotion, oxidative stress and endothelial dysfunction. All the THPs tested demonstrated significantly reduced responses in these *in vitro* assays when compared to 3R4F. The findings suggest these products have the potential for reduced health risks. Further pre-clinical and clinical assessments are required to substantiate the risk reduction of these novel products at individual and population levels.

1. Introduction

Although cigarette smoking is an established cause of severe health problems including cardiovascular disease, chronic obstructive pulmonary disease, and cancer (US Department of Health and Human Services, 2010, 2014), tobacco products continue to be consumed on a global scale. In 2014, the number of cigarettes smoked worldwide was estimated at 5.8. trillion (Eriksen et al., 2015). The scientific consensus is that most smoking-related diseases are caused not by nicotine but by toxicants that are either present in the tobacco itself or formed in the smoke during the combustion and pyrolysis process (Farsalinos and Le Houezec, 2015).

In recent years, the case has been made for the role of tobacco harm reduction, defined as “decreasing total morbidity and mortality, without completely eliminating tobacco and nicotine use” (Stratton et al., 2001), in decreasing the health burden of tobacco use (McNeill et al., 2015). The US Food and Drug Administration (FDA) has published guidance on assessing the harm reduction potential of a “modified risk tobacco product” (MRTP) with the demonstration of either decreased toxicant exposure or reduced health risks (FDA 2012).

Products that heat tobacco or nicotine have been marketed in various formats, from early cigarette-shaped products that are used similarly to conventional cigarettes but do not burn down (RJ Reynolds Co., 1988), to electrically heated modular systems that bear a resemblance to everyday items such as pens and memory sticks, and larger cylindrical or rectangular devices (WHO, 2014). These electronic nicotine delivery systems (or ENDS), especially electronic cigarettes (e-cigarettes or “vapour products”) and more

* Corresponding author.
E-mail address: damien_breheny@bat.com (D. Breheny).

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recently tobacco-heating products (THPs), have increased markedly in popularity over the past decade (European Commission, 2015; Schoenborn and Grindi, 2015; West et al., 2015). The design and operation of ENDS means that, compared with conventional cigarettes, they have the potential for tobacco harm reduction on both an individual and population level.

Electronic vapour products deliver an aerosol, obtained by heating either tobacco or an "e-liquid", that users inhale (WHO, 2014). The aerosol from vapour products forms very differently to cigarette smoke. In cigarettes, the tobacco is burnt and pyrolysed to form smoke containing more than 6500 compounds (Rodgman and Perfetti, 2013); around 150 of these are thought to be toxicants and related to disease (Fowles and Dybing, 2003). In THPs, a plug of blended or processed tobacco, similar to that used in conventional cigarettes, is heated to temperatures much lower (<400 °C) than those in the tip of a burning cigarette (~900 °C). However, this lower temperature is sufficient to vapourise the nicotine and other volatile compounds in the tobacco into an inhalable stream, but not high enough to burn the tobacco and form combustion compounds. Vapour products such as e-cigarettes do not contain tobacco, and thus the aerosol does not contain tobacco-related toxicants; the main constituents of the e-liquid by volume are nicotine (if present), propylene glycol (with or without vegetable glycerol), and flavouring agents (WHO, 2014). However, impurities and flavour degradation products may be present in both e-liquids and aerosol emissions.

In many countries, the marketing of novel tobacco products is subject to regulatory approval, obtained by submitting details of the design, performance, and impact of the device on users and non-users. Some regulators are considering the need for substantial data packages of pre-clinical, clinical, and population studies to support the regulatory approval of novel products. In the United States, such studies already form part of a pre-market tobacco authorisation (PMTA) and for MRTP applications (FDA, 2012). For counties in the European Union, such studies might become a requirement under updates of the Tobacco Products Directive (EU, 2014). The Tobacco Product Assessment Consortium (TobPRAC) have developed a conceptual framework, highlighting key tests and reference products required to demonstrate reduced harm and product stability by chemical, toxicological and human studies, in order to guide the evaluation of new tobacco products (Berman et al., 2015). Lowe et al., 2015 have also proposed a four-phase framework, describing a series of pre-clinical (analysis of chemical emissions and a series of *in vitro* biological and toxicological tests), clinical and population studies, for the scientific evaluation of new tobacco and nicotine products with a focus on THPs and e-cigarettes.

In vitro tests provide a means of screening new product innovations in an efficient manner, in terms of both time and cost. 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 high-throughput *in vitro* screening technologies. The National Research Council's, "Toxicity Testing in the 21st Century: A Vision and a Strategy" (NRC, 2007) outlines approaches using advances in molecular biology, biotechnology, *in vitro* and computational science to help evaluate the health risks of consumer products and safety assessment of chemicals. The assays selected for assessment of products in this study were based on *in vitro* endpoints that have well-established links to cigarette smoke exposure and smoking related disease.

This paper is the second of a two-part study (Poynton et al., 2017). The focus here was to use a range of *in vitro* laboratory methods to assess the toxicological and biological responses following exposure to aerosol from novel tobacco products including: a novel hybrid tobacco product (hybrid) which operates at near ambient temperature, a commercially available THP (c-THP) and a prototype THP (p-THP). The novel hybrid tobacco product delivers warm aerosol generated from an electronic vapour product that is combined with a natural flavour tobacco note released from a segment of tobacco (Fig. 1). The characteristics of the hybrid tobacco product and its aerosol chemistry are described in Part 1 of this study (Poynton et al., 2017). The operating mechanism of the novel hybrid product contrasts with that of the c-THP and p-THP, which are electrical heating devices that produce tobacco aerosol by heating a tobacco rod at up to 350 °C and 240 °C, respectively (Fig. 1).

We have focused on a number of *in vitro* tests based on classical toxicological and biological endpoints relevant to smoking related disease, adopted from the assessment framework of Lowe et al. (2015), which is summarised in Fig. 2. These tests fall into two categories, '*in vitro* regulatory assays' and '*in vitro* models of disease', which fulfil part of Phase 1 (stewardship science) and Phase 2 (toxicant exposure reduction) of the assessment framework, respectively. The results obtained are compared with effects after exposure to cigarette smoke from a scientific research reference cigarette, 3R4F.

2. Materials and methods

2.1. Reference cigarette, hybrid tobacco product and THPs

The Kentucky 3R4F reference cigarette is a US-blended king-sized tobacco product with a cellulose acetate filter and an International Organisation for Standardization (ISO) tar yield of 9.4 mg/cigarette in approximately nine puffs. It is one of the most well-characterised reference cigarettes in terms of its blend composition, physical construction and mainstream smoke toxicant (e.g., harmful and potentially harmful constituents [HPHC]) yields, see Supporting Information Table S1 (Roemer et al., 2012; University of Kentucky, 2015).

The hybrid tobacco product is a button-operated electronic vapour device consisting of a USB-rechargeable 650-mAh Li-ion battery and an integrated circuit power controller, onto which a closed system cartomiser is attached, and has been described in detail by Poynton et al. (2017).

The prototype THP (p-THP) consists of a research tobacco rod heated in a portable oven to 240 °C (Forster et al., 2015), while the commercial THP (c-THP) is a rechargeable device that heats a tobacco consumable to 350 °C. The latter product is commercially available in a few countries (e.g. Japan).

2.2. Machine puffing regimen

The test exposure matrices were generated by using defined machine puffing regimens. For the 3R4F cigarette, mainstream smoke was generated following the Health Canada Intense (HCI) puffing regime: 55 mL puff volume, 2 s puff duration, 30 s puff interval, with a bell-shaped puff profile; 100% vent blocking (Health Canada, 1999). A modification of this HCI regime was used for the p-THP and c-THP, where vent blocking was not possible but all other parameters were the same as standard HCI. The hybrid tobacco product was puffed using a 55 mL puff volume, 3 s puff duration and 30 s puff interval, using a rectangular flow profile puff.

2.3. Generation and characterisation of test matrices for assessment

Three different test matrices were used for *in vitro* assessments: total particulate matter (TPM), whole aerosol (WA), and aqueous aerosol extract (AqE). The test matrices and the appropriate