# The assessment of a range of next generation tobacco and nicotine products using pre-clinical *in vitro* tools.

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## INTRODUCTION

There has been significant growth in the number of next generation products available as alternatives for smokers, predominantly e-cigarettes but also novel tobacco heating products (THPs) that heat rather than burn tobacco. As these products do not burn tobacco (or in the case of e-cigarettes, do not contain tobacco), the toxicant profile of their aerosols may be greatly reduced in comparison to cigarettes and therefore they may have potential to be reduced risk products. This paper will describe the *in vitro* assessment of an e-cigarette, a "hybrid" THP and a prototype THP, comparing the results relative to the 3R4F reference cigarette.

## METHODS

The products used for these studies were the 3R4F reference cigarette (University of Kentucky, USA), a prototype THP, a hybrid THP (see Poster #112) and an e-cigarette (Vype® ePen). All test articles were generated under Health Canada Intense (HCI) smoking regime (55/2/30, vents blocked on 3R4F). No vent holes are present on the novel products.

**Smoke extract production**

Conventional 3R4F reference cigarettes, a prototype THP, a hybrid THP or Vype® E-pen devices were smoked or activated on a Borgwaldt-KC RM20H smoking machine. Particulate matter (TPM) extracts were prepared as previously described[1]. Aerosol aqueous extract (AqE) was generated by bubbling the smoke from a single cigarette, or 10 puffs of vapour (THP heated to 180°C), or 10 x 3 second activations (hybrid THP/Vype®) through 20mL of cell culture medium.

**Ames bacterial reverse mutation assay**

Product particulate matter exposures were conducted according to the principles of OECD 471, however utilising only *S. typhimurium* strain TA98+S9. For product whole aerosol (WA) exposures, the Ames assay was modified as previously described[2]. Briefly, TA98 was exposed to WA using a Vitrocell VR AMES 4 stainless steel module (D) smoke engine, in a scaled-down 35mm plate format for a period of 64 minutes.

**Cytotoxicity assay**

Human bronchial epithelial cells (NCI-H292) were exposed to WA at the air-liquid interface (ALI) for a period of 1 hour, using a Borgwaldt RM20S smoking machine (Borgwaldt KC, Hamburg, Germany). Following exposure, cytotoxicity was assessed using Neutral Red Uptake as previously described[3].

**Gamma H2AX assay**

Human bronchial epithelial cells (BEAS-2B) were exposed to WA at the ALI for a maximum of 3 hours[4]. Immunostaining and a high content screening (HCS) approach using the Cellomics ArrayScan® Vti platform was used to determine the frequency of DNA double strand breaks.

**Bhas42 cell transformation assay**

Bhas 42 cells (v-Ha-ras-transfected Balb/c 3T3 clone A31-1-1) were treated with TPM from test products using the promoter protocol[5]. Cells were treated for 7 days to particulate matter with media change every 3 days, followed by a 7 day recovery. Plates were scored and results evaluated as previously described[6].

**Antioxidant depletion**

NCI-H292 cells were exposed to AqE for a period of 4 hours. The ratio of reduced to oxidised glutathione (GSH:GSSG) was analysed in lysates using the GSH:GSSG Glo™ assay kit (Promega, UK).

**Endothelial cell migration assay**

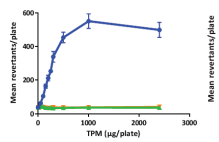
Human umbilical vein endothelial cells were grown to confluency, "wounded" using a pipette tip and exposed to various AqE for 22 hours in 24 well ImageLock plates (Essen Instruments, Ann Arbor, MI, USA). Cell migration was quantified by measuring the closure of the "wound" using an Incucyte time lapse video camera and software (Essen Instruments, Ann Arbor, MI, USA).

**Pro-inflammatory cytokine secretion**

MucilAir™ primary airway epithelial cultures were exposed to whole aerosols (1/20 dilution ratio) for 4x five minute intervals with 30 minutes rest between each exposure, using a Borgwaldt RM520 smoke engine. Cytokines were quantified with the Meso Scale Discovery V-plex 30 cytokine and 3-Plex MMP kits, according to manufacturers instructions (Gaithersburg, USA).

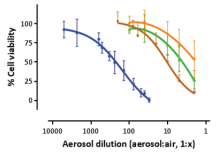
## RESULTS

### Mutagenicity

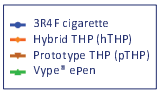



Novel product particulate matter did not induce mutations, whereas cigarette smoke demonstrated a positive dose response

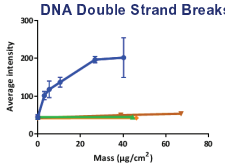
### Cytotoxicity




Compared to cigarette smoke, novel products demonstrated significantly lower cytotoxicity

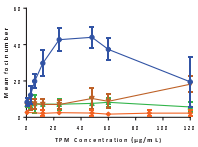



### DNA Double Strand Breaks



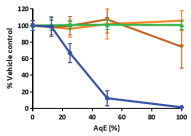

Novel product whole aerosol did not induce mutations or DNA double strand breaks across all doses, whereas cigarette smoke demonstrated a positive dose response

### Cell Transformation



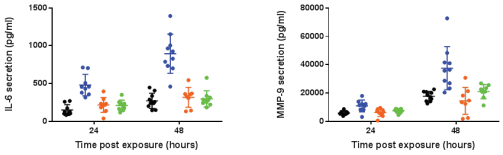

hTHP and Vype® particulate matter did not induce transformation of Bhas initiated cells, whereas cigarette smoke demonstrated a positive dose response. pTHP was elevated at the top dose

### Antioxidant Depletion



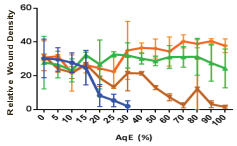

hTHP and Vype® did not cause antioxidant depletion, whereas cigarette smoke demonstrated a positive dose response. pTHP induced slight depletion at the highest dose

### Pro-Inflammatory Cytokine Secretion




MucilAir™ expression of IL-6 and MMP-9 was significantly elevated following exposure to cigarette smoke. Novel product aerosols were not significantly different from air controls (shown in black)

### Endothelial Migration




hTHP and Vype® did not impair endothelial cell migration, whereas cigarette smoke and pTHP demonstrated a positive dose response

## CONCLUSIONS

- Under these test conditions using a number of *in vitro* assays, next generation tobacco and nicotine products demonstrated reduction in responses compared to a reference 3R4F cigarette, indicating a potential to be reduced risk versus cigarettes
- Additional research including clinical and population studies are needed to substantiate disease relevant risk reduction in populations

**References**

1. The use of a novel tobacco-substitute sheet and smoke dilution to reduce toxicant yields in cigarette smoke. KG McAdam et al. Food Chem Toxicol. (2011) 49(8): 1684-96.
2. A method for assessment of the genotoxicity of mainstream cigarette-smoke by use of the bacterial reverse-mutation assay and an aerosol-based exposure system. J. Kilford et al. Mutat. Res. (2014) 769: 20-28.
3. Evaluation of an air–liquid interface cell culture model for studies on the inflammatory and cytotoxic responses to tobacco smoke aerosols. D. Azzopardi et al. Toxicol In vitro. (2015) 29 (7): 1720–1728
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6. Cigarette Smoke-induced Morphological Transformation of Bhas 42 Cells In Vitro. D Weisensee et al. ATLA (2013) 41: 181-189.

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## Abstract

The concept of the risk continuum was first presented in 2012 to rank the reduced risk potential of a range of tobacco and nicotine products relative to cigarettes. The US Food and Drug Administration (FDA), is currently the only national regulator that has provided draft guidance with which to assess the harm reduction potential of novel tobacco products via their Modified Risk Tobacco Products draft guideline. Building on this guidance, we recently published an integrated assessment framework which proposed the use of pre-clinical, clinical and population studies to assess the reduced risk potential of new products at the individual and population level.

There has been significant growth in the number of smokers currently using next generation products, predominantly e-cigarettes but also novel Tobacco Heating Products (THPs) that heat rather than burn tobacco. As these products do not burn tobacco (or in the case of e-cigarettes, do not contain tobacco), the toxicant profile of their aerosols is greatly reduced in comparison to cigarettes and therefore hold promise as reduce risk products.

This paper will describe the in vitro assessment of an e-cigarette, a "hybrid" THP and a prototype THP and compare the results relative to conventional cigarettes. The novel products were assessed across a range of in vitro toxicological assays specifically measuring mutagenicity and cytotoxicity and showed greatly reduced responses relative to cigarettes. Following this, products were assessed using human-cellular based in vitro assays that model some of the key events for smoking related diseases such as COPD and CVD. These tests across all assays used indicated reduced responses relative to cigarettes. Using Mucilair™ reconstituted lung epithelial tissue cultures, we further assessed functional COPD key events in response to whole aerosol exposure from these novel products and observed substantial reductions in responses for the THP, hybrid THP and e-cigarette relative to cigarettes.

Based on these in vitro assessments, the next generation tobacco and nicotine products tested demonstrated a potential to be reduced risk versus cigarettes, however a series of clinical and population studies measuring the longer terms effects of these new products on consumers is required to substantiate disease relevant risk reduction.

## Acknowledgements

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## Related Publications

1. The use of a novel tobacco-substitute sheet and smoke dilution to reduce toxicant yields in cigarette smoke. McAdam KG, Gregg EO, Liu C, Dittrich DJ, Duke MG, Proctor CJ. Food Chem Toxicol. (2011) 49(8):1684-96.

2. A method for assessment of the genotoxicity of mainstream cigarette-smoke by use of the bacterial reverse-mutation assay and an aerosol-based exposure system, J. Kilford J, Thorne D, Payne R, Dalrymple A, Clements J, Meredith C, Dillon D. Mutat. Res. (2014) 769 : 20–28.

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5. An international validation study of a Bhas 42 cell transformation assay for the prediction of chemical carcinogenicity. Sakai A, Sasaki K, Hayashi K, Muramatsu D, Arai S, Endou N, Kuroda S, Poth A, Bohnenberger S, Kunkelmann T, Asakura M, Hirose H, Ishii N, Mizuhashi F, Kasamoto S, Nagai M, Pant K, Bruce SW, Sly JE, Yamazaki S, Umeda M, Tanaka N. Mutat. Res . (2011) 725 : 57-77.

6. Cigarette Smoke-induced Morphological Transformation of Bhas 42 Cells In Vitro. Weisensee D, Poth A, Roemer E, Conroy LL, Schlage WK. ATLA (2013) 41 : 181-189

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