NEXT GENERATION PRODUCTS

Assessment of the Cardiovascular impact of e-vapour and heated tobacco products using New Approach Methodologies

ACT 45th Annual Meeting November 17-20th 2024, Submission ID: 1830780

Matthew Stevenson[1]*, Fiona Chapman[1], Edgar Trelles Sticken[2], Sarah Jean Pour[2], Roman Wieczorek[2], Luuk de Haan[3], Linda Gijzen[3], Matthias Gossman[4], Bettina Lickiss[4], Liam Simms[1]

[1]Imperial Brands PLC, 121 Winterstoke Road, Bristol, BS3 2LL, UK, [2]Reemtsma Cigarettenfabriken GmbH, An Imperial Brands PLC company, Albert-Einstein-Ring-7, D-22761, Hamburg, Germany, [3]Mimetas B.V., Biopartner Building 5, De Limes 7, 2342 DH Oegstgeest, The Netherlands, [4]InnoVitro GmbH, Artilleriestraße 2, 52428, Jülich, Germany

*Corresponding author's e-mail: Matthew.Stevenson@impbrands.com

FIND OUT MORE

INTRODUCTION

Imperial Brands understands that smoking is a cause of serious disease in smokers including lung cancer, heart disease and emphysema. The greatest risk of smoking related diseases comes from burning tobacco and inhaling smoke containing around 7,000 chemicals. While science suggests that nicotine is addictive and not risk-free, Public Health experts worldwide have concluded that it is the toxicants in cigarette smoke generated by burning tobacco, and not nicotine, which is the primary cause of smoking-related disease. Tobacco Harm Reduction (THR) refers to strategies designed to reduce the health risks associated with tobacco smoking. Next Generation Products (NGP), like Heated Tobacco Products (HTP) and E-Vapour Products (EVP), deliver nicotine without burning tobacco so have the potential to play a role in THR.

There is a need for methods to assess the impact of products such as EVPs and HTPs on smoking-related disease endpoints, but also to detect any potential new hazards which could occur with these, and other novel innovative products. Here we present data obtained comparing in vitro cardiovascular models' responses to EVPs and HTPs, to those from a cigarette in a variety of *in vitro* New Approach Methodologies (NAMs). We exposed the various NAMs, to cigarette smoke and aerosol (EVP & HTP) extracts bubbled through phosphate buffered saline or cell medium, as an appropriate systemic exposure technique.

METHODS

2.1 Test Articles

- 1R6F Reference Cigarette (University of Kentucky)
- Heated Tobacco Product (HTP), "PULZE" with "iD" tobacco stick
- E-vapour product (EVP), blu pod-based system with tobacco flavour pod

Both NGPs were obtained from the EU market.

2.2 Smoke / Aerosol Extract Generation method

Smoke and aerosol from test products were generated with a Vitrocell VC10s (Vitrocell, Munich, Germany) smoking machine. Smoke or aerosol extracts were prepared by bubbling the sample aerosol into 3 in-line Impingers each containing 10 mL of either Phosphate Buffered Saline (PBS) solution or cell media (See Figure 1). A total stock solution of 30 mLs per test article was used.

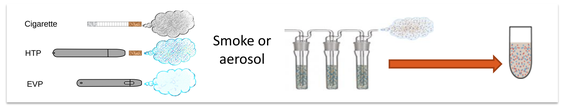

Figure 1: Bubbling smoke/vapour exposure system

Trapped nicotine was quantified within the aerosol and smoke bubbled PBS (bPBS) or Media (bMed) samples, using liquid chromatography with tandem mass spectrometry (LC-MS/MS) with an AB Sciex API 6500 QTRAP (SCIEX, Framingham, MA, USA) using nicotine-d4 as the internal standard.

2.3 Biological Assessment

Scratch Wound Assay: Human umbilical vein endothelial cells (HUVEC) were scratch wounded and exposed to different concentrations of bubbled medium extracts (bMed). A WoundMaker™ device was used to conduct the scratch in the cell monolayer. Wound healing is measured as the relative wound density (RWD) over time as calculated by image-based data evaluation. An iterative scanning analysis over 30h was performed with the IncuCyte ZOOM®1.

Cardiomyocyte contractility and cytotoxicity: iCell hiPSC-derived Cardiomyocytes were exposed to the test articles and assessed with the CardioExcyte 96™ and FLEXcyte 96™ systems2. The FLEXcyte 96 system, cultures cardiomyocytes on ultra-thin silicone membranes, mimicking the mechanical environment of native human heart tissue. Rhythmic contractions deflect the membranes, quantified by capacitive distance sensing. Parameters analysed were contractile force (AMP), beat rate (BR), beat rate regularity (BRR), rising time (RT), falling time (FT). The time range of observation was 0 - 24 hours post-test article exposure. The CardioExcyte 96 is an impedance-based system where cardiomyocytes are cultured on gold electrodes, enabling label-free and continuous quantification of electrical impedance as a measure of monolayer integrity.

High Content Screening (HCS): Human coronary artery endothelial cells (HCAECs) were exposed to increasing concentrations of bubbled medium extracts (bMed) for either 2 or 24h and cellular live staining with subsequent fixation was applied. Assessment utilised the HCS device (Cellomics array scan XTI); a staining kit to detect generation of reactive oxygen species (CellRox™ green) and an inhouse method for quantification of GSH based on the property of monochlorobimane to bind reduced GSH. The subsequent impact of 1mM N-Acetylcysteine (NAC - a ROS scavenger) supplementation was also determined.

Organ-on-a-Chip: HCAECs were cultured on an OrganoPlate®2-lane chip (Mimetas BV) combined with THP-1 monocytes under flow conditions3. THP-1 monocytes were exposed to bPBS, with the resulting conditioned medium being added to the HCAEC vessels, and the following endpoints assessed: Oxidative stress (monochlorobimane added to cultures), ICAM expression (immunofluorescent readout), monocyte adhesion (immunofluorescent readout) and inflammatory marker release in medium (ProCartaPlex Inflammation 20-plex (Thermo Fisher Scientific).

RESULTS

3.1 Dosimetry: Nicotine levels in bubbled PBS / medium extracts

- To simulate the compounds absorbed into the blood stream following inhalation of cigarette smoke or aerosol from HTP or EVPs, we employed PBS or cell media as an exposure technique. Cell media has the benefits of exposing cells to higher levels of smoke / aerosol components present (up to 97% w/w inclusion), whereas PBS is limited to a maximum concentration in some techniques of up to 10% w/w. However, PBS has the benefit of chemical simplicity and can be used in a wide variety of tests without significant levels of method optimisation.
- Both Media and PBS, captured supraphysiological levels of nicotine from all three test items, with the nicotine recorded across bubbling runs for: PBS = 1R6F: 217µg/ml, HTP: 166µg/ml & EVP: 181µg/ml, and media = 1R6F: 225µg/ml , HTP: 333µg/ml, & EVP: 504.4µg/ml). NOTE: physiological levels of nicotine in Cigarette, HTP and EVP adult users ranged from 5 – 20 ng/ml.

3.2 Biological Results

3.2.1 Scratch Wound Assay

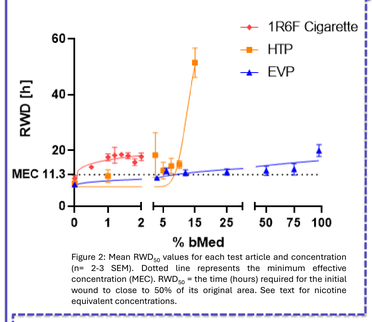

Figure 2: Mean RWD_{50} values for each test article and concentration (n= 2-3 SEM). Dotted line represents the minimum effective concentration (MEC). RWD_{50} = the time (hours) required for the initial wound to close to 50% of its original area. See text for nicotine equivalent concentrations.

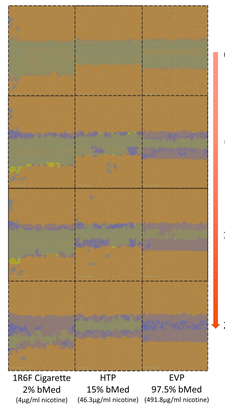

Figure 3: Representative phase contrast images of HUVEC cells taken at 0h, 6h, 12h and 24h post wounding following exposure to 1R6F Cigarette Smoke, Pulze aerosol and blu 2.0 aerosol bubbled mediums. Colour key: Orange = HUVEC Cells, Olive = Initial scratch wound mask. Purple = HUVEC cell Confluence mask, BLUE = cell free area occurring due to cell migration during wound healing

- 1R6F bMed caused a statistically significant increase of the RWD_{50} value compared to the control after treatment with 1, 1.2, 1.4, 1.6 and 2% bMed which is equivalent to 2, 2.4, 2.8, 3.2 and 4µg/ml nicotine. The concentration required to exceed the MEC was calculated with 0.25% bMed.
- HTP bMed caused a statistically significant increase of the RWD_{50} value compared to the control after treatment with 15% bMed which is equivalent to 46.3µg/ml nicotine. The concentration required to exceed the MEC was calculated with 9.3% bMed.
- blu 2.0 bMed caused a statistically significant increase of the RWD_{50} value compared to the control after treatment with 97.5% bMed which is equivalent to 491.8µg/ml nicotine. The concentration required to exceed the MEC was calculated with 39.92% bMed.

3.2.2 Cardiomyocyte contractility and cytotoxicity

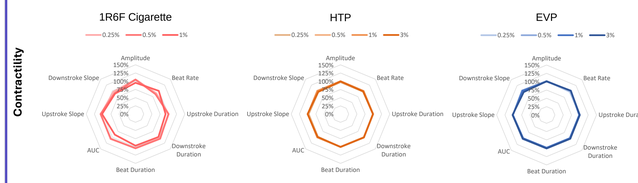

Figure 4: Contractility analysis (FLEXcyte 96) for the three test articles to determine any functional cardiotoxicity. 3% 1R6F bPBS caused extensive cytotoxicity and therefore didn't elicit any response in the parameters and is not shown in the figure, n=6.

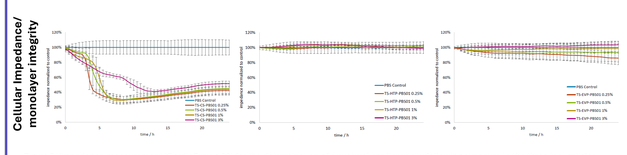

Figure 5: Impedance measurement (CardioExcyte 96) for the determination of structural cardiotoxicity following exposure to the three test articles, n=6.

- **Contractility**: The 1R6F bPBS had a significant negative impact on the contractility of cardiomyocytes at concentrations of 1% (equivalent to 2.17µg/ml nicotine) and 3% (equivalent to 6.51µg/ml nicotine). While the effect was transient at 1%, the cells ceased beating after approx. 300 min at 3%. HTP and EVP bPBS had little or no effect on the contractility of cardiomyocytes up to the top tested concentration of 3% (equivalent to 4.98 and 5.42µg/ml nicotine for HTP and EVP respectively).
- No arrhythmic events were observed for any of the test samples
- **Cellular Impedance:** All concentrations of 1R6F bPBS induced a significant decrease in electrical impedance within the first 5 hours after exposure, indicating a strong cardiotoxic effect. Both the HTP and EVP bPBS had a negligible impact on impedance, indicating a low cardiotoxic effect.

3.2.3 High Content Screening

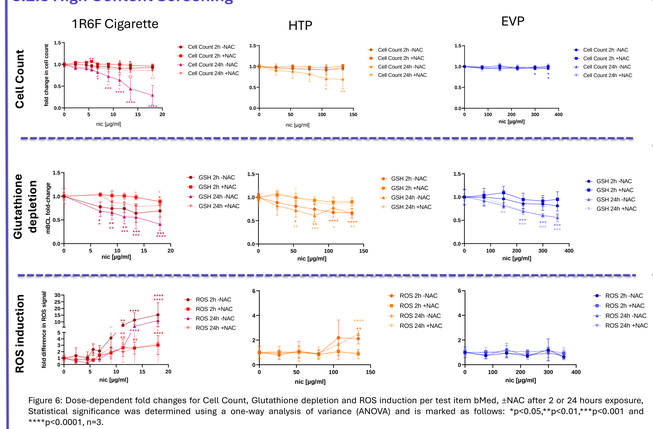

Figure 6: Dose-dependent fold changes for Cell Count, Glutathione depletion and ROS induction per test item bMed, ±NAC after 2 or 24 hours exposure, Statistical significance was determined using a one-way analysis of variance (ANOVA) and is marked as follows: *p<0.05,**p<0.01,***p<0.001 and ****p<0.0001, n=3.

- **Cell Count:** No significant disruptions to cell count were observed for the 2h treatment for any of the products (±NAC). Following 24h of exposure severe toxicity was seen for 1R6F cigarette bMed but this was nullified upon NAC addition. Mild disruptions to cell count were observed at 24h treatment with HTP or EVP bMed. These were nullified with NAC.
- **Glutathione depletion:** All products were seen to deplete GSH at the 24h time points. The 1R6F bMed was the most potent test article, with it having a MEC of 7.3µg/ml and 25.3µg/ml nicotine (24h±NAC respectively). HTP and EVP bMed depleted GSH at 6 - 37-fold higher concentrations.
- **ROS induction:** Reproducible ROS induction was seen in both treatments 2h and 24h for 1R6F bMed (>11.3µg/ml), with NAC largely reversing this. In contrast, HTP induced this at markedly higher concentrations (133.4µg/ml) than 1R6F, whilst EVP didn't induce any ROS up to 355.7µg/ml .

3.2.4 Organ-on-a-Chip

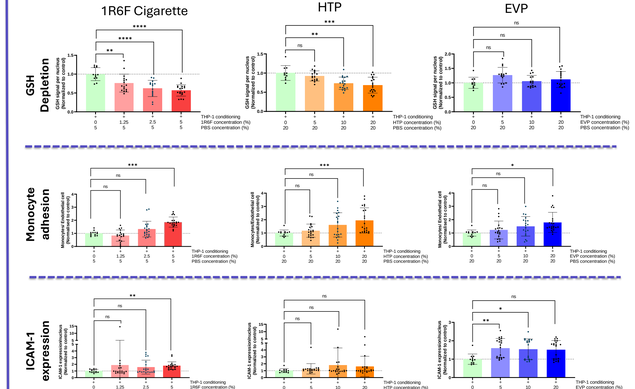

Figure 7: GSH depletion (4h exposure), Monocyte adhesion (24h exposure) and ICAM-1 expression (24h exposure) in endothelial cells. Statistical significance was determined using a one-way analysis of variance (ANOVA) and is marked as follows: *p<0.05,**p<0.01,***p<0.001 and ****p<0.0001, n=2.

- **GSH Depletion:** 1R6F bPBS conditioned medium elicited dose dependant reductions in glutathione levels, indicating increasing levels of oxidative stress (Figure 7). This trend was also observed for the HTP (at four-times higher concentrations), whilst no significant changes were observed for EVP.
- **Monocyte adhesion:** Dose dependent increases in monocyte adhesion were observed for all three test articles. These were only significant at the respective highest concentrations applied 1R6F: 5% bPBS vs HTP/EVP: 20% bPBS.
- **ICAM-1 expression:** There were no clear trends in ICAM-1 (a cell surface protein involved in the inflammatory response) expression, potentially due to high variability in responses.
- **Inflammatory markers (data not shown):** 1R6F was the most potent test article, followed by HTP and EVP bPBS. 1R6F bPBS induced 18 markers, notably, e-selectin, p-selectin, ICAM-1, IFN-alpha and IL-6.

CONCLUSIONS

In summary, the 1R6F cigarette bubbled extracts induced several biological effects in a variety of NAMs, including cellular dysfunction, oxidative stress, disrupted contractility, immune cell recruitment and proinflammatory effects. In contrast, HTP and EVP extracts displayed markedly reduced or no effects at much higher exposures. The NAMs used for this assessment sensitively detected toxicity from 1R6F cigarette at low exposures and could also differentiate between the two NGPs at the higher, and therefore less potent concentrations.

These results add to the weight-of-evidence that the tested EVP and HTPs have the potential to offer a harm reduced alternative to smoking cigarettes under these conditions of test and the potential to make a meaningful contribution to tobacco harm reduction.

REFERENCES

[1] Chapman F, Sticken ET, Wieczorek R, Pour SJ, Dethloff O, Budde J, Rudd K, Mason E, Czekala L, Yu F, Simms L, Nahde T, O'Connell G, Stevenson M. Multiple endpoint in vitro toxicity assessment of a prototype heated tobacco product indicates substantially reduced effects compared to those of combustible cigarette. Toxicol In Vitro. 2023 Feb;86:105510. doi: 10.1016/j.tiv.2022.105510.

[2] Goßmann, M., Frotscher, R., Linder, P., Neumann, S., Bayer, R., Epple, M., Staat, M., Artmann, A. T., & Artmann, G. M. (2016). Mechano-Pharmacological Characterization of Cardiomyocytes Derived from Human Induced Pluripotent Stem Cells. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology, 38(3), 1182–1198

[3] Chapman F, de Haan L, Gijzen L, Strijker W, Sticken ET, Pour SJ, Wieczorek R, Haberstroh F, Otte S, Nahde T, Simms L, Stevenson M. Optimisation of an in vitro human cardiovascular model on-a-chip for toxicological assessment of nicotine delivery products. Front Toxicol. 2024 Jun 13;6:1395670. doi: 10.3389/ftox.2024.1395670.

IMPERIAL BRANDS SCIENCE