An *in vitro* approach to e-cigarette toxicity testing

EUROTOX September 2018

IMPERIAL BRANDS

SCIENCE

Visit our Scientific Research website
www.imperialbrandsscience.com

L. Simms[1], L. Czekala[1], M. Stevenson[1], G. Phillips[1], R. Tilley[1], K. Rudd[1], T. Walele[1]
1 Imperial Brands PLC, 121 Winterstoke Road, Bristol, BS3 2LL, UK

1. INTRODUCTION

When the National Academies of Sciences published "Toxicity Testing in 21st Century: A Vision and a Strategy" a new toxicological paradigm was created, focusing on the use of human derived cells lines/tissues and the disruption of key cellular pathways (NRC, 2007). In keeping with these principles, Imperial Brands has sought current assays for harm reduction assessment of our products, with toxicological testing framework shown in Figure 1.

The study explored the potential use of three in vitro assays utilising human primary cells. The three *in vitro* platforms studied were High Content Screening (HCS); the use of primary cells (BioMap®); and a 3D reconstituted human lung model exposed to e-liquid aerosol. To determine the suitability of each test system, either base e-liquids or e-cigarette aerosol were use.

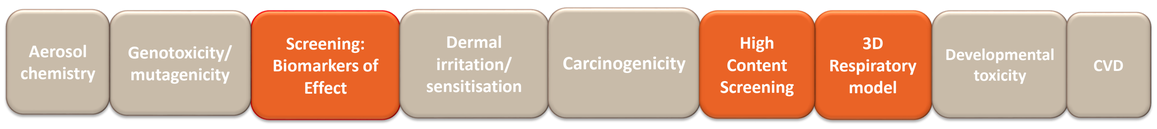
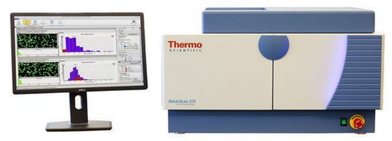


Figure 1: *In vitro* testing framework for harm reduction assessment

- HCS was conducted with human primary NHBE cells exposed to neat e-liquids for up to 24 hours, using fluorescent staining to study a range of cell health markers at the same time.
- The BioMAP®, Diversity PLUS product consisted of 12 primary human cell-based systems designed to model different tissues and disease *in vitro* (see Table 2). Cells were stimulated with a combination of biological proprietary factors (e.g. cytokines, growth factors, mediators, etc.) to recreate the multi-component signalling networks associated with disease states.
- Human 3D lung cells (EpiAirway™, MatTek Corp.) were exposed to e-liquid aerosol and a reference cigarette (3R4F) to study cellular effects using a wide range of cellular markers.

2. HIGH CONTENT SCREENING OF E-LIQUIDS



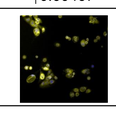
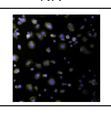
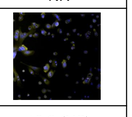
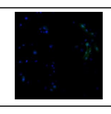
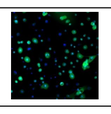
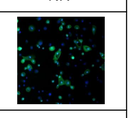
Test concentrations:

- Normal Human Bronchial Epithelial (NHBE) cells were exposed to 0.0313-3% e-liquid (Base liquid 1.2% [w/w] nicotine and 2.4% [w/w] nicotine) or 3R4F smoke condensate (CSC) at 0.001-0.02% for 24 hours.

Results:

- ≤ 3% possible osmolality effects of e-liquids (Gonzalez-Suarez *et al.,* 2017).
- In general, toxicological responses were observed for 3R4F at much lower concentrations than for e-liquids. Cytotoxicity was observed at very low concentrations of CSC compared to e-liquids.
- Increasing nicotine concentration in base liquid formulation lowered the minimum effect concentration (MEC) for certain cellular endpoints (e.g. cell cycle arrest), shown in Table 1.

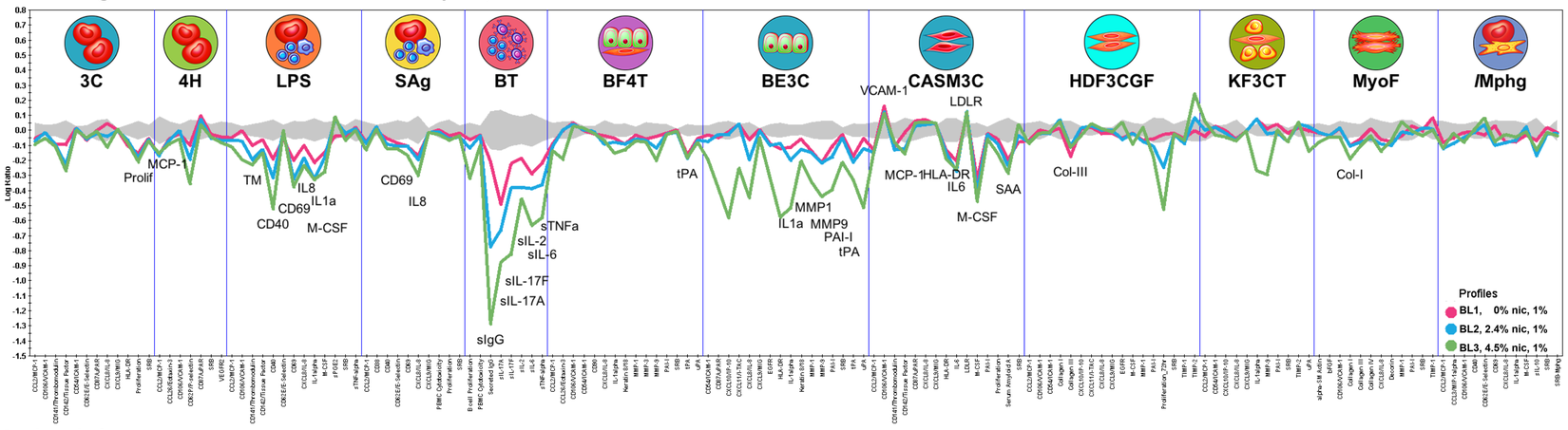
Table 1: MEC that significantly crossed vehicle control threshold (%)

	Minimum effective concentration (MEC) that significantly crosses vehicle control threshold (%)		
	3R4F (0.001 - 0.02%)	**BL 1.2% (0.0313 – 3%)**	**BL 2.4% (0.0313 – 3%)**
Cell count	↓0.001	NR	↓2.1
Cell Cycle Arrest	↑0.00129	↑1.69	↑0.718
Cell Membrane Permeability	↑0.001 (NS)	↑0.345 (NS)	↑0.149 (NS)
Caspase 3/7 Intensity	↑0.001	NR	NR
NF-κB	↑0.00467	NR	NR
			
Mitochondrial Mass	↑0.00582	NR	↓0.8 (NS)
Mitochondrial Membrane Potential (Δψm)	↓0.00533	NR	NR
Oxidative Stress	↓0.0049	NR	NR
Glutathione Content	↓0.00801	NR	NR
			
Cellular ATP	↓0.00454	↓1.55 (NS)	↓1.02

NR No response observed, NS Fit not statistically significant,
↑↓ Direction of response

3. EFFECTS OF INCREASING CONCENTRATIONS OF NICOTINE IN BASE E-LIQUIDS ON THE BIOMAP®

Figure 2: The effects of Base liquid, ± 2.4% and 4.5% nicotine concentrations added to BioMAP at 1% concentration



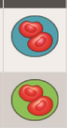
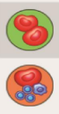
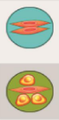
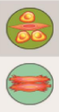
Methodology:

- BioMap® Plus Panel utilizes 12 human primary cell based systems to simulate various key cellular pathways within the human body (Table 2).
- Tested base e-liquids (BL) all contained 50:50 propylene glycol (PG) and vegetable glycerine (VG), with varying nicotine content: 0% [BL1], 2.4% [BL2] and 4.5% [BL3]. All e-liquids were tested at eight concentrations (0.031-4%). Solutions were added directly to the cell media.

Results:

- BL with different nicotine concentrations added to cell media at concentrations above 0.5% led to a characteristic fingerprint of biomarkers and dose response relationship. Increasing concentrations of nicotine led to an exaggeration of the fingerprint profile in selected cell panels, above that for base e-liquid itself.
- The cell systems which produced the most notable response was the BT and BE3C cell systems.
- The BioMap Plus Panel is a useful screening tool to give an indication of where to focus further research and is not diagnostic of disease.

Table 2: Biomap® 12 primary human cell-based systems

System Name	Icon	Cell	Disease
3C		Venular endothelial cells	Cardiovascular Disease, Chronic Inflammation
4H		Venular endothelial cells	Allergy, Asthma, Autoimmunity
LPS		Peripheral blood mononuclear cells, Venular endothelial cells	Cardiovascular Disease, Chronic Inflammation
SAg		Peripheral blood mononuclear cells, Venular endothelial cells	Autoimmune Disease, Chronic Inflammation
BT		B cells, Peripheral blood mononuclear cells	Allergy, Asthma, Autoimmunity, Oncology
BF4T		Bronchial epithelial cells, Dermal fibroblasts	Allergy, Asthma, Fibrosis, Lung Inflammation
BE3C		Bronchial epithelial cells	COPD, Lung Inflammation
CASM3C		Coronary artery smooth muscle cells	Cardiovascular Inflammation, Restenosis
HDF3CGF		Dermal fibroblasts	Chronic Inflammation, Fibrosis
KF3CT		Dermal fibroblasts, Keratinocytes	Dermatitis, Psoriasis
MyoF		Lung fibroblasts	Chronic Inflammation, Fibrosis, Matrix Remodeling, Wound Healing
/Mphg		Macrophages, Venular endothelial cells	Cardiovascular Disease, Chronic Inflammation, Restenosis

4. USE OF 3D RECONSTRUCTED LUNG MODELS EXPOSED TO E-CIGARETTE AEROSOL AND CIGARETTE SMOKE AT AIR-LIQUID INTERFACE

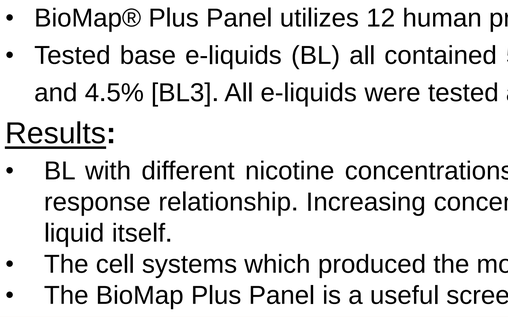
Methodology:

- E-cigarette aerosol generated using the CORESTA Recommended Method N°81 (55mL/3s/30s; square wave puff profile). Conventional cigarette smoke was generated using the Health Canada Intense method (55mL/2s/30s).
- Morphological evaluation conducted using H&E staining and EpiAirway™ tissue viability was assessed 24 hours after exposure using the MTT assay (MatTek Corp.). Cell membrane integrity was assessed via measurement of the tight junctions using trans-epithelial electrical resistance (TEER). The concentration of 8-isoprostane in conditioned media was measured using a competitive ELISA kit.

Results:

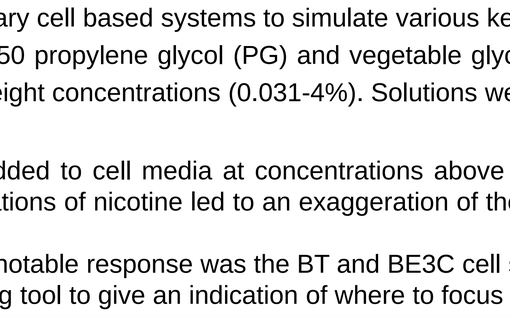
- E-cigarette aerosol up to the highest dose of 400 puffs did not alter tissue viability or barrier function compared to matched air control.
- 8-Isoprostane (a biomarker of oxidative stress and antioxidant deficiency) results demonstrated no oxidative stress responses in samples exposed to either base e-liquid with 2.4% nicotine under the conditions of test.
- CS significant decreased tissue viability and barrier function and caused a significant increase in oxidative stress response.

Figure 3: Tissue viability



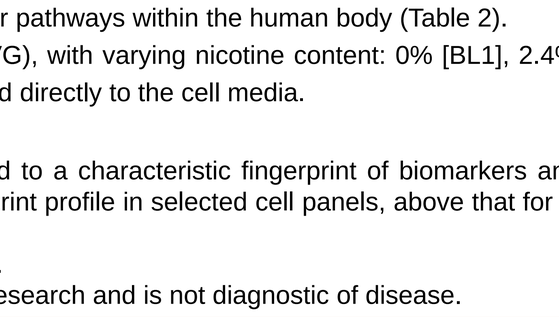
Tissue viability declined to 85% and 27% following exposure to 27 puffs and 45 puffs of cigarettes, respectively. Tissues remained 100% viable with exposure to the base e-liquid aerosol up to 400 puffs. **p-value ≤ 0.05*

Figure 4: TEER



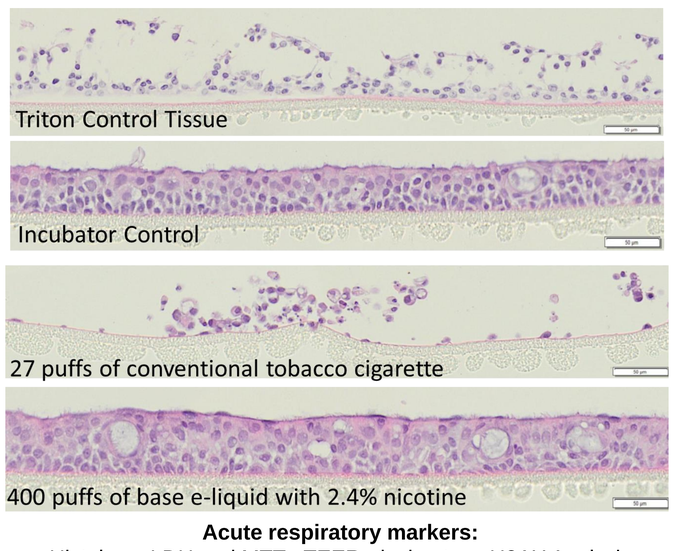
Exposure to cigarette smoke, 27 and 45 puffs, significantly reduced TEER to ~18 Ω*cm² and 3.7 Ω*cm² respectively (1.7% and 0.5% of the matched air-exposed tissues). Base e-liquid aerosol did not impair barrier function up to the highest dose tested. **p-value ≤ 0.05*

Figure 5: Oxidative Stress Response



Cigarette smoke produced significantly increased amounts of 8-isoprostane in a dose-dependent manner. 8-isoprostane levels did not alter for samples exposed to base e-liquid aerosol up to 400 puffs. **p-value ≤ 0.05*

Figure 6: H&E staining



Acute respiratory markers:
Histology; LDH and MTT; TEER; dosimetry; γH2AX Analysis (DNA double strand breaks); TUNEL (Apoptosis), oxidative stress (8-isoprostane), IL-6, IL-8 and MCP-1

5. CONCLUSIONS

- Neat e-liquids and aerosol at physiologically relevant concentrations did not generally elicit a toxicological response in the range of *in vitro* assays tested. Our findings are consistent with other researchers.
- The *in vitro* assays explored here are a quick screening tool for assessing the toxicity of e-liquids and add to a Weight of Evidence (WoE) approach.
- We believe these *in vitro* assays have the potential to greatly contribute to our current knowledge of e-liquid ingredients and aerosols and should form part of a larger weight of evidence approach including clinical studies for the assessment of this category of products.

REFERENCES

- NRC (2007) Toxicity testing in 21st Century- A vision and a strategy https://www.nap.edu/catalog/11970/toxicity-testing-in-the-21st-century-a-vision-and-a
- Gonzalez-Suarez *et al.,* (2017) App In vitro Toxicol 3(1): 41-55.
- CORESTA recommended method 81 https://www.coresta.org/sites/default/files/technical_documents/main/CRM_81.pdf