

### A PRODUCT STEWARDSHIP ASSESSMENT STRATEGY FOR E-LIQUIDS

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This presentation is the opinions of the author and is for discussion purposes only, it may not necessarily reflect the opinions of Imperial Brands plc.

## IMPERIAL BRANDS ADOPT A SCIENTIFIC APPROACH TO ASSESSING PRODUCTS WITHOUT TESTING ON ANIMALS

We have a duty to understand how our products interact and behave with consumer health, and any potential risks that may materialise:

- Consumer safety is key
- Regulatory compliance for market of sale

Imperial Brands PLC does not commission or conduct research involving animals and would not undertake such research unless formally required to do so by governments or by recognised regulatory authorities.

IB has an Alternatives to Animal Testing approach which involves using human relevant *in vitro* and *in silico* techniques

- These additional assays provide a weight of evidence and can help clarify results of routine assays.
- Our in vitro approach has been endorsed by PETA science



"We are a proponent of good science and sparing animals from toxicity tests, as Imperial has done." Dr Andreas Stucki, PETA Science Consortium International

### **PRODUCT STEWARDSHIP ASSESSMENTS STRATEGIES**

- Various manufacturers have published their EVP aerosol assessment strategies
- The only independent scientific group to write one, is the UK's Committee of Toxicology (COT)
- The guidance suggests a desk-based review, using quantitative risk assessment, history of use and Thresholds of Toxicological concern to assess individual ingredients.
- We broadly follow this framework however focus on the *in vitro* testing of the liquids/aerosols from the device they will be marketed in



Framework for risk assessment of flavouring compounds via inhalation exposure





# BIOLOGICAL TESTING; PART OF A SCIENTIFIC ASSESSMENT FRAMEWORK (SAF)

A multi-stage, multiyear testing and research programme designed to evaluate the potential harm reduction of each of our NGPs relative to combustible cigarettes.





https://imperialbrandsscience.com/







## **DESK BASED TOXICOLOGICAL ASSESSMENT ACTIVITIES**

Qualified Toxicologists determine quality and purity of the individual ingredients of an e-liquid, using a range of techniques:

- Review information from suppliers
- Existing scientific literature and Read-Across
- In Silico Predictions (e.g. Lhasa DEREK, ToxTree, OECD Toolbox)
- If these initial desk-based investigations are satisfactory we move the novel e-liquid into *in vitro* testing (liquid and aerosol) and HPHC analysis of the whole aerosol









## IN VITRO ASSESSMENT OF E-LIQUIDS AND WHOLE AEROSOLS

E-Liquids are initially assessed neat to determine the formulation mixture biological activity:

Neutral Red Uptake Assay	Human bronchial epithelium Beas-2B cells
Ames assay	5 Salmonella strains +/-S9
In Vitro Micronucleus	Human lymphoblastoid TK6 cells

To determine the impact of e-liquid aerosolization, cells are also exposed to whole aerosol in the following techniques:

Neutral Red Uptake Assay	Human bronchial epithelium Beas-2B cells
Ames assay	5 Salmonella strains +/-S9
In Vitro Micronucleus	Chinese hamster lung V79 cells) at ALI





### IN VITRO ASSESSMENT OF E-LIQUIDS AND WHOLE AEROSOLS CONT.

For smoke / aerosol generation, we use the "Smoke Aerosol Exposure In Vitro System" (SAEIVS), an inhouse system to enable cells to be exposed at the ALI to whole smoke or aerosol.

Fig A and B : Schematic of SAEIVS:



For Ames, the bacterial suspension is bubbled with smoke /aerosol prior to plating to increase the potential for exposure to the bacteria

Fig C: Bubbling of concentrated bacterial suspension for Ames test:





# EXAMPLE RESULTS COMPARING A POD-BASED E-CIGARETTE TO 3R4F REFERENCE CIGARETTE IN



Rudd, K., et al., (2020). Chemical composition and in vitro toxicity profile of a pod-based e-cigarette aerosol compared to cigarette smoke. Applied In Vitro Toxicology, 6(1), pp.11-41.



### FURTHER MECHANISTIC INSIGHTS CAN BE OBTAINED FOR GENOTOXIC/ MUTAGENIC RESPONSES



Czekala, L., et al., (2021). The in vitro ToxTracker and Aneugen Clastogen Evaluation extension assay as a tool in the assessment of relative genotoxic potential of e-liquids and their aerosols. *Mutagenesis*, 36(2), pp.129-142.



# HIGH CONTENT SCREENING FOR ADDITIONAL ENDPOINTS: INFLAMMATION (NFKB)



Fig 1: HCS images with Etoposide treated cells for the red and green fluorescence intensity indicating the level of  $\gamma$ H2AX (circle regions; red) and amount of Nf $\kappa$ B respectively (circle and ring regions, green).



Table 1 Summary of MEC and AC50 responses for 3R4F CSC and different e-liquids \*

						Hig	h Content S	creening - B	iologia	al Res	ponse Hea	t Map						
	3R4F (0.001- BL 0% (0.5, 1.0, 0.02%) 3%)			BL 1.2% 3'	(0.0313– %)	BL 2.4% (0.0313- 3%)		Blueberry 2.4% (0.0313-3%)				cco 2.4% 13 – 3%)	Menthol 2.4% (0.0313-3%)			Vanilla 2.4% (0.0313 – 3%)		
	MEC	AC50%	MEC	AC50%	MEC	AC50	MEC	AC50	N	1EC	AC50	MEC	AC50	MEC	AC50		MEC	AC50
Cell count	10.001	0.008	NR	NR	NR	NR		>31	1	1.06	1.78	1.36	2.08	10.36	0.885		<b>↓1.48</b>	1.89
Cell Cycle Arrest	10.001	>0.005	NR	NR	<u></u> †1.69	2.0	<u></u> ↑0.718	0.982	10	.377	0.598	↑0.363	0.548	10 0551	0.096		10.406	0.573
Cell Membrane Permeability	10.001	0.021	NR	NR	10.345 (NS)	>3ł (NS)	↑0.149	>31	†0	.783	1.22	†1.52	2.05	↑0.659	0.855		†1.64	2.06
Caspase 3/7 Intensity	10.001	>0.015i	NR	NR	NR	NR	NR	NR	10	.596	>3ł	†0.652	>3ł	10.106	>2i		<b>†1.17</b>	>3ł
NF-KB		>0.02 <del>1</del> (NS)	NR	NR	NR	NR	NR	NR	ť	1.12	1.86	†1.86	2.54	†0. <del>6</del> 3	0.991		† <b>1.</b> 9	2.42
Mitochondrial Mass	10.006	>0.021	NR	NR	NR	NR	10.8 (NS)	>3ł (NS)		NR	NR	†1.88	>3 <del>1</del>	†0.88	>1ŧ		†2.98	>3ł
Mitochondrial Membrane Potential (Δψm)		0 012	NR	NR	NR	NR	NR	NR	to	.865	1.05	10.88	1.94	10.689	1.36		↓1.16	2.32
Oxidative Stress	10.005	900.0	NR	NR	NR	NR	NR	NR	1	NR	NR	↓0.836	1.61	10.833	1.63	İĽ	↓1.08	1.96
Glutathione Content	10.008	0.008	NR	NR	NR	NR	NR	NR	ţ,	1.35	1.65	↓1.77	2.14	↓0.735	0.816		<b>↓1.9</b>	2.15
Cellular ATP		>0.02i	12.59	>3	↓1.55 (NS)	>3ł (NS)	↓1.02	1.82	ŤO	.952	1.31	.↓1.76	1.76	10.602	0.773		↓1.62	1.71

\*Czekala, L., et al., 2019. High Content Screening in NHBE cells shows significantly reduced biological activity of flavoured e-liquids, when compared to cigarette smoke condensate. Toxicology in vitro, 58, pp.86-96. NHBE Normal human bronchiole epithelial cells

### **RECAP OF PROCESS TO ASSESS E-LIQUIDS**



Ingredients and materials of sufficient quality/on any negative lists?

Compliance with limits of purity and any contaminants/any country specific regulatory restrictions



'Deskbased' risk assessment using existing scientific information



Screening of Ingredients for CMRS activity

Screening for Carcinogenic, Mutagenic, Reproductive and Respiratory/skin Sensitisation properties



Laboratory testing using a regulatory battery of tests

> Assessment of the VP mixture, as both liquid and whole



XV

Any Additional testing based on results e.g., Toxys, HCS, 3D tissues

Explore ToxTracker/HCS to look at possible mechanism of actio Decision

Considering all data including smoke chemistry data and comparison to historical products



### **CONCLUSIONS:**

- We hold a robust toxicological assessment process, utilising a variety of different alternative to animal testing approaches (desk based, in silico and in vitro)
- We use expert toxicologists to assess our materials and products, to ensure only suitable products are released
- We will continue to develop our Toxicological techniques to facilitate quicker and better decision making for our NGPs

frontiers	DRGNAL RESEARCH publicks 11 February 2020 drs 10.2089-0023 /2170
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	Use of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes to Predict the Cardiotoxicity Potential of Next Generation Nicotine Products
	Liem Simms <sup>1</sup> *, Fan Yu <sup>1</sup> , Jessica Palmer <sup>2</sup> , Kathyn Rudd <sup>1</sup> , Edgar Trelles Sticken <sup>2</sup> , Roman Wieczonk <sup>2</sup> , Fiona Chapman <sup>1</sup> , Lukasz Czekala <sup>1</sup> , Matthew Stevenson <sup>1</sup> and Grant O'Connel <sup>1</sup>
	<sup>1</sup> Impetial Brands PEC, Bristol, United Ringdom, <sup>4</sup> Stemina Biomarker Discovery Inc., Madison, WI, United States, <sup>3</sup> Reemtern Cigarestembolson GmbH, Hamburg, Germany
OPEN ACCESS Edited by: Archear Stark, PRA Sense Constitu- interactional a V, Germany Reviewed Constitu- interactional a V, Germany R, M, Liber States Jamph Mancpaols, Physiciane Conveillen br Palepointable Medicine Libert States	Contrasting the cognitive strating is mainly black that basis for surdivances of second product sections, the conditionation product strational end of second product product strational end of the second product strating the second product strating and the second product strating and second product strating and second products and second products and second product strating and second products and second product strating and second products and second second products and second second products and second
"Carrespondence: Liam Simma Jam simma@uk.imptab.com	aims were to investigate the cardiotoxicity potential of NGPs compared to cigarettes, in addition to nicotine. To accomplish this, hiPSC-CM were exposed to smoke or aeroso bubbled PBS samples: reference cigarette (1RBP: three variants of HTP: and three DP
Specially section: This article was submitted to in Vitro Tosicology, a section of the journal Promises in Tosicology Received: 26 July 2021	bbbbbs / hbb dat/pbb/fbbbs and bbbbbs / hbb dat/bbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbb

cotine). Both 1R6F and HTF I four predictive metabolites the assay up to the maxim concentration tested (10% bPRS). Nicotine tested on its own was predicted to have cardiotoxic potential at concentrations greater than 80 µg/mL, which is higher than expected physiological levels associated with combustible cigarette smoking. The application of this rapid screening assay to NGP research and the associated findings adds to the weight-of-evidence indicating that NGPs have a tobacco harm reduction potential when compared to combustible clearettes. Additionally, this technique was shown to be sensitive and robust for the assessment of different NGPs and may be considered as part of a larger overall scientific framework for NGP assessments. ds: cigarette, NGP, nicotine, cardiotoxicity, hiPSC-CM, in vitro, NAM

### APPLIED IN VITRO TOXICOL Volume 8, Number 1, 2022 Mary Ann Liebert, Inc. DOI: 10.1089/aixt.2021.0020



Demonstrates Reduced In Vitro Toxicity Compared with Tobacco Snus and Combustible Cigarette Smoke

Fan Yu<sup>1</sup> Kathryn Rudd<sup>1</sup> Sarah Jean Pour.<sup>2</sup> Edoar Trelles Sticken.<sup>2</sup> Ole Dethloff.<sup>2</sup> Roman Wieczorek: Thomas Nahde,<sup>9</sup> Liam Simms,<sup>1</sup> Fiona Chapman,<sup>1</sup> Lukasz Czekala, Matthew Stevenson,<sup>1</sup> and Grant O'Connell<sup>1</sup>

Introductions: Toheco hum modecian (THR)) appression one of the most provisiong public health policies with continued public interviewing metalic hum (THR) a ready. However, the Moster (THR) are as recent THR product innovation wallable in a graving number of countries as a potentially less humidi alterna-tive to traditional obscience products. Materialia and Methadic: In this study, we compared the in vitro biological activity of two commercially available TTRN, nose sum product, and combastile capitert to make a three to intro toxicological activity available TTRN, nose sum product, and combastile capiter to make a three in vitro toxicological activity available

take [NRU], in vitro micronucleus [IVM], and Ames test). An extraction method for TFNPs and snus in sphate-buffered saline based on the Intern ational Organization of Standardization 10993-12 standard to as prophene control with biological activity is also presented. sees the in vitro biological activity is also presented. total particle matter (TPM) induced a statistically signif-icant positive response in all three in vitro assays. By contrast, the TPMPs and suss extracts were negative in both

s and IVM assays, and demonstrated weak cytotoxicity in the NRU assay compared with TPM, under the ns of test. When using the HepG2 cell line in the NRU assay, it was possible to differentiate between ENP and sums extracts, with only sums extracts resulting in a measurable  $BC_{20}$  response. First initial preclinical *in vitro* taxicity assessment of TSNPs compared with both tobacco snus and musuable cigarette smoke indicates that the tested TSNPs have a substantially reduced *in vitro* toxicity activity mpared with traditional tobacco products. Given this initial data, both further scientific exploration and public club discussions are unsurement. in these products are warranted. icity, genotoxicity, in vitro, tobacco harm reduction, tobacco-free nicotine pouches

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Imperial Brands PLC, Bristol, United Kingdom. <sup>10</sup>-meteres Company, Hamburg, Germany.

Rollmani Caparessension tomes, or an Anna Liebert, Inc. This Open Access article is distributed under the term O Fars Yu et al., 2022; Published by Mary Anna Liebert, Inc. This Open Access article is distributed under the term Common Attribution Noncommercial Licenses [ICC-BY-NCI] (http://creativecommercy.org/licenses/by-nci/40/) which provide the organization and the term of 
Acute electronic vapour product whole aerosol exposure of 3D human bronchial tissue results in minimal cellular and transcriptomic responses when compared to cigarette smoke

### Gary Phillips<sup>1</sup>©, Lukasz Czekala<sup>1</sup>, Holger P Behrsing<sup>2</sup>, Khalid Amin<sup>3</sup>, Jessica Budde<sup>4</sup>, Matthew Stevenson<sup>1</sup>, Roman Wieczorek<sup>4</sup>©, Tanvir Walele<sup>1</sup>, and Liam Simms<sup>1</sup>

Abstract The advanced sequence products (19%) possibles to increase involvedage and with advances is ead advances The advances balong on the companional increases there is this scenarios predices and the predict Interest where a special and exclusion of the companional increases there is the scenarios prediction of the prediction of the other and exclused possibility harm where compared to ciperate surveils. To better understand the possibility and hadds directs associated with exposurs to DFM exact softwards where associated the cubility of advances and the advances and exclusion of the other system (PistalAVII<sup>1</sup>) following a single adv-optionatic exposure to cigrates amount and the advances increase there are other of DFM exact All examptions, circle (10% for the Advance) and the advances of the advances of DFM exact All examptions, circle advances of the Advances of DFM exact All examptions, circle advanced to the BM examptions of DFM exact All examptions, circle advanced advances of DFM exact All examptions, circle advanced to the BM examptions, circle advanced and the advances of DFM exact All examptions, circle advanced to the BM examptions, circle advanced and the advances of DFM exact All examptions, circle advanced advances of DFM exact All examptions, circle advances of DFM ex ur products (EVPs) continues to in and percent active area (XAA), tram epithelial electrical resistance (TEER), histology) and cytokine release were assessed at 4- and 48- hours following recovery from air, EVP aerosol (8.4% VIV: myblu<sup>TH</sup> blueberry favour, 2.4% nicotine) and 3R4F smoke (3.5% VIV: exposure). No pathological charges were observed at either recovery time point from an exposure. Air and EVP aerosol exposure had no effect on CBF, %AA nor TEER at 48 hours. Exposure to cigarette smoke resulted in a decrease in TEER, an increase in CBF and the release of proinflammatory cytokines at both recovery time ints. Although the number of significantly expressed genes was minimal following exposure to EVP aerosol, exposure t IR4F smoke resulted in a significant upregulation of several disease relevant pathways. These data provide evidence that following an acuse exposure to EVP aerosol there is significantly less damage to lung cells in culture than the equivalent nicotine based, dose of cigarette smoke.

Keywords Aerosol, e-cigarette, transcriptomics, inflammation, harm reduction, nicotine

Data received 15 October 2020 accented 28 December 2020 Group Science and Regulatory Affairs, Imperial Brands PLC, Bristol, United Kingdom

### <sup>1</sup>Respiratory Toxicology, Institute for In Vitro Sciences, Inc., Gatherburg, HD, USA <sup>1</sup>University of Minasota Medical Center, Minnapolis, MN, USA Cigarette smoke is a dynamic and complex mixture of par-ticulates and gaues and contains more than 6000 chemicala, it some of which have been classified as hummful or potentially harmful by the US Food and Drug Administration.<sup>2</sup> Currently there are more than 1 billion cigarette smokers worldwide<sup>2</sup> and epidemiological studies have linked exposure to cigarette smoke with diseases such as cancer,

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### Multi-endpoint analysis of human 3D airway epithelium following repeated exposure to whole electronic vapor product aerosol or cigarette smoke

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structures (World Health Organization, 2020). Public health bad-report that smoking is a cause of serious diseases in smokers tional Center for Chronic Disease, 2014; World Health role to play in tobacco harm reduction (THR) for adult smokers would otherwise continue to smoke. Public Health England exit that EVPs are at least 95% less harmful than combustible cigar



### Original Manuscript

The in vitro Tox Tracker and Aneugen Clastogen Evaluation extension assay as a tool in the assessment of relative genotoxic potential of e-liquids and their aerosols

### Lukasz Czekala<sup>1</sup>, Fiona Chapman<sup>1</sup>, Liam Simms<sup>1</sup>, Kathryn Rudd dgar Trelles Sticken², Roman Wieczorek², Lisa Maria Bode², Jutta Pani Nynke Moelijker<sup>3</sup>, Remco Derr<sup>2</sup>, Inger Brandsma<sup>3</sup>, Giel Hendriks<sup>2,\*</sup>, Matthew Stevenson<sup>1</sup> and Tanvir Walele<sup>1</sup>

roup Science and Regulatory Affairs, Imperial Brands PLC, 121 Winterstoke Road, Bristol BS3 2LL, UK, 'Reemtam Infabrikan BribH, an Imperial Brands PLC Company, Albert-Einstein-Ring-7, D-22761 Hamburg, Germany and xys B.V., Robert Boyleweg 4, 2333 CG Leiden, The Netherlands

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In vitro (genoltoxicity assessment of electronic vapour products (EVPs), relative to intertie, currently uses assays, including the micronucleus and Ames tests. Whilst informativ induction of a finite endpoint and relative risk posed by test articles, such assays could banef ation. The ToxTracker and Aneugen Clar in indicate the activation of reporters associated with (geno)toxicity, including DNA damag xidative stress, the p53-related stress response and protein damage. Here, we tested for the ifferent effects of a selection of neat e-liquids, EVP aerosols and Kentucky reference 1R6F cigarette moke samples in the ToxTracker assay. The assay was initially validated to assess whether These analysis is the sectored as a sector of the sector o arity in vitro. E-liquid nicotine contant did not affect resp ing nicotine alone only induced an oxidative stress res promy income the two processing is a quick, informative serve is appropriate the constraint of a constraint of the two processing of the constraint of the c moking alternative) products, including EVPs

### ACKNOWLEDGMENTS

- Matthew Stevenson, Scientific Substantiation and Engagement
- Biological Toxicology Laboratory and Toxys for conducting the *in vitro* studies
- Co-authors on the various manuscripts cited in this presentation