Next Generation Products Induce Lower Biological Activity than Combusted Cigarettes Using BioMAP® System of Human Primary Cell Based Co-Cultures

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1. INTRODUCTION

Smoking is a cause of serious disease. Whilst Public Health authorities recommend cessation as the best option, for those unwilling or uninterested to quit smoking, the use of electronic cigarettes are recommended (McNeil et al., 2015). To assess the harm reduction potential of NGPs, we compared the Kentucky Reference Cigarette (3R4F) to three NGPs (IQOS, referred to as (THP); IFUSE (HYB) and *myblu* (EVP)) in 12 cell culture systems using the Eurofins DiscoverX BioMap Diversity PLUS® testing panel. Diversity PLUS testing utilises 148 biomarker readouts (7-17 per cell system) selected for therapeutic and biological relevance which can be used to predict disease outcomes or specific drug effects in the 12 cell systems. These cell systems utilise human primary cells which were validated using chemicals with known mechanisms of action, contained in the Eurofins Discovery reference data base. This system has previously been used in pharmaceutical industry for drug discovery and has a broad applicability to a variety of diseases. As an extension to the BioMAP biomarkers, Eurofins DiscoverX used data mining to identify new mechanisms of toxicity linked to human adverse events by identifying groups of key biomarkers altered by reference compounds. These phenotypic signatures (Toxicity Signatures) were then applied to the BioMAP results to look for potential toxicity mechanisms.

2. MATERIALS AND METHODS

2.1 Test Samples (all commercially available)

- Kentucky Reference Cigarettes (3R4F)
- IQOS Tobacco Heated Product (THP), 0.5 mg/stick nicotine
- IFUSE Hybrid Product (HYB); 1.8 % nicotine
- *myblu*™ Tobacco flavour; 1.6% nicotine (EVP)

2.2 Smoke / Aerosol Extract Generation

Table 1: Smoke and Aerosol Generation Regime

3R4F	HTP	EVP HYB
ISO Intense puffing regime		ISO Vaping regime
• 55 mL puff volume • 2s duration • 30s intervals • Modifications for HTP		• 55 mL puff volume • 3s duration • 30s interval

- Smoke or aerosol extracts were prepared by bubbling the sample aerosol into three in-line impingers each containing 10 mL Phosphate Buffered Saline (PBS) (Figure 1).

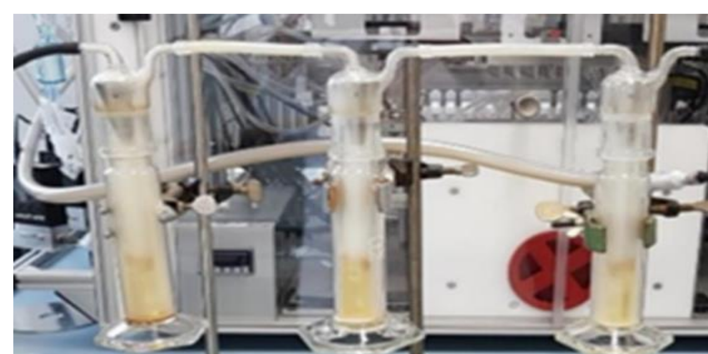


Figure 1: Bubbling smoke/ aerosol exposure system

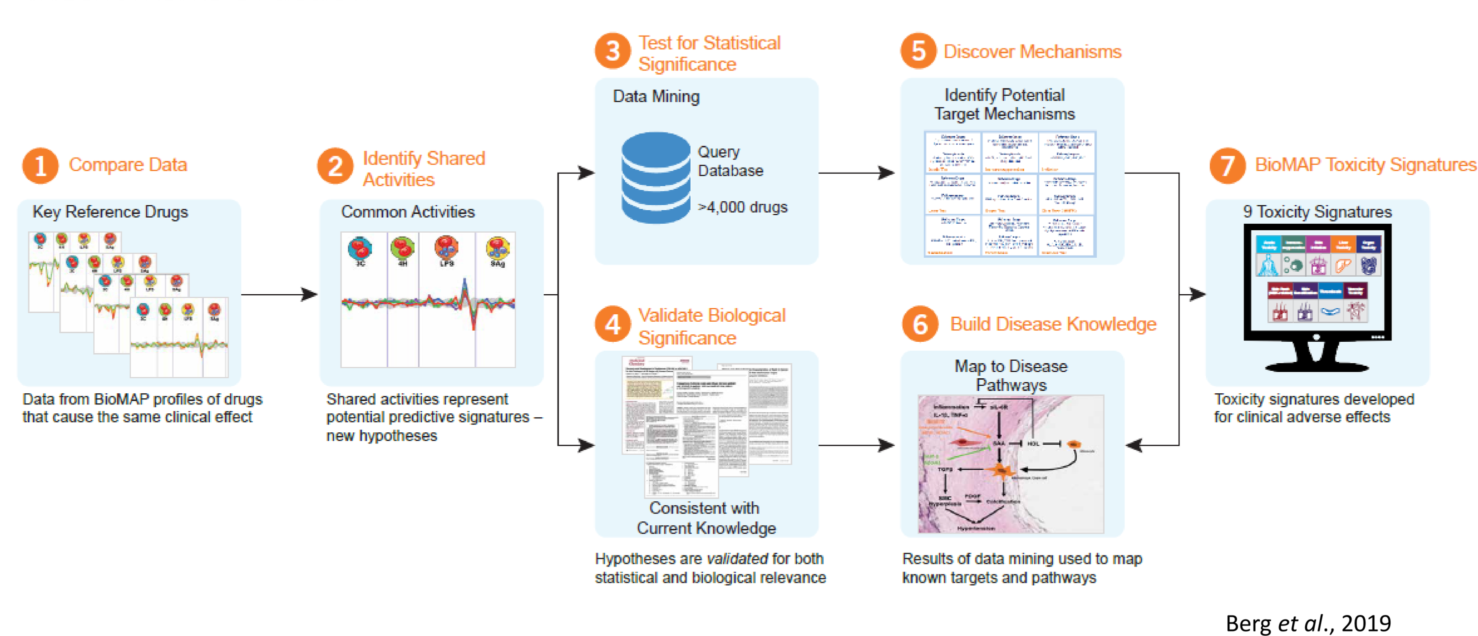
- A total stock solution of 30 mL per test article was used: 1.8 puffs per ml for 3R4F and 4 puffs per ml for all NGPs.
- Exposure concentrations were 0.12, 0.25, 0.5 and 1% for all PBS samples for each of the 12 BioMap cell systems (Table 2).
- Nicotine and carbonyls trapped in fresh PBS samples were quantified using validated LC-MS/MS and HPLC-DAD methods respectively.
- For nicotine measurement, the internal standard Nicotine-d4 was used. For carbonyl determination 2,4-Dinitrophenyl hydrazine (DNPH) was used and the carbonyl-DNPH derivates were detected. A mean of three replicates per sample are summarized in Table 4.

2.3 Table 2: Diversity Plus cell systems, disease relevance and biomarkers end points

System Name	Icon	Cell	Disease	Biomarker Readouts
3C		Venular endothelial cells	Cardiovascular Disease, Chronic Inflammation	MCP-1, VCAM-1, TM, TF, ICAM-1, E-selectin, uPAR,IL-8, MIG, HLA-DR, Proliferation, SRB
4H		Venular endothelial cells	Asthma, Allergy, Autoimmunity	MCP-1, Eotaxin-3, VCAM-1, P-selectin, uPAR, SRB, VEGFRII
LPS		Peripheral blood mononuclear cells, Venular endothelial cells	Cardiovascular Disease, Chronic Inflammation	MCP-1, VCAM-1, TM, TF, CD40, E-selectin, CD69, IL-8, IL-1α, M-CSF, sPGE2, SRB, sTNFα
SAg		Peripheral blood mononuclear cells, Venular endothelial cells	Autoimmune Disease, Chronic Inflammation	MCP-1, CD38, CD40, E-selectin, CD69, IL-8, MIG, PBMC Cytotoxicity, Proliferation, SRB
BT		Peripheral blood mononuclear cells, B cells	Asthma, Allergy, Oncology, Autoimmunity	B cell Proliferation, PBMC Cytotoxicity, Secreted IgG, sIL-17A, sIL-17F, sIL-2, sIL-6, sTNFα
BF4T		Bronchial epithelial cells, Dermal fibroblasts	Asthma, Allergy, Fibrosis, Lung Inflammation	MCP-1, Eotaxin-3, VCAM-1, ICAM-1, CD90, IL-8, IL-1α, Keratin 8/18, MMP-1, MMP-3, MMP-9, PAI-1, SRB, tPA, uPA
BE3C		Bronchial epithelial cells	Lung Inflammation, COPD	ICAM-1, uPAR, IP-10, I-TAC, IL-8, MIG, EGFR, HLA-DR, IL1α, Keratin 8/18, MMP-1, MMP-9, PAI-1, SRB, tPA, uPA
CASM3C		Coronary artery smooth muscle cells	Cardiovascular Inflammation, Restenosis	MCP-1, VCAM-1, TM, TF, uPAR, IL-8, MIG, HLA-DR, IL-6, LDLR, M-CSF, PAI-1, Proliferation, SAA, SRB
HDF3CGF		Dermal fibroblasts	Fibrosis, Chronic Inflammation	MCP-1, VCAM-1, ICAM-1, Collagen I, Collagen III, IP-10, I-TAC, IL-8, MIG, EGFR, M-CSF, MMP-1, PAI-1, Proliferation_72hr, SRB, TIMP-1, TIMP-2
KF3CT		Keratinocytes, Dermal fibroblasts	Psoriasis, Dermatitis, Skin Biology	MCP-1, ICAM-1, IP-10, IL-8, MIG, IL-1α, MMP-9, PAI-1, SRB, TIMP-2, uPA
MyoF		Lung fibroblasts	Fibrosis, Chronic Inflammation, Wound Healing, Matrix Remodeling	a-SM Actin, bFGF, VCAM-1, Collagen-I, Collagen-III, Collagen-IV, IL-8, Decorin, MMP-1, PAI-1, TIMP-1, SRB
/Mphg		Venular endothelial cells, Macrophages	Cardiovascular Inflammation Restienosis, Chronic Inflammation	MCP-1, MIP-1α, VCAM-1, CD40, E-selectin, CD69, IL-8, IL-1α, M-CSF, sIL-10, SRB, SRB-Mphg

- Eurofins Discovery includes Toxicity Signatures as an additional analysis tool, using the BioMap traces as reference products of known mechanisms of action. Combining the biomarker changes of the reference products provided a biomarker signature. The summary of their signature identification process is summarised below.

Figure 2: Toxicity Signature Analysis development using reference compounds with known mechanisms of action



Berg *et al.*, 2019

3. RESULTS

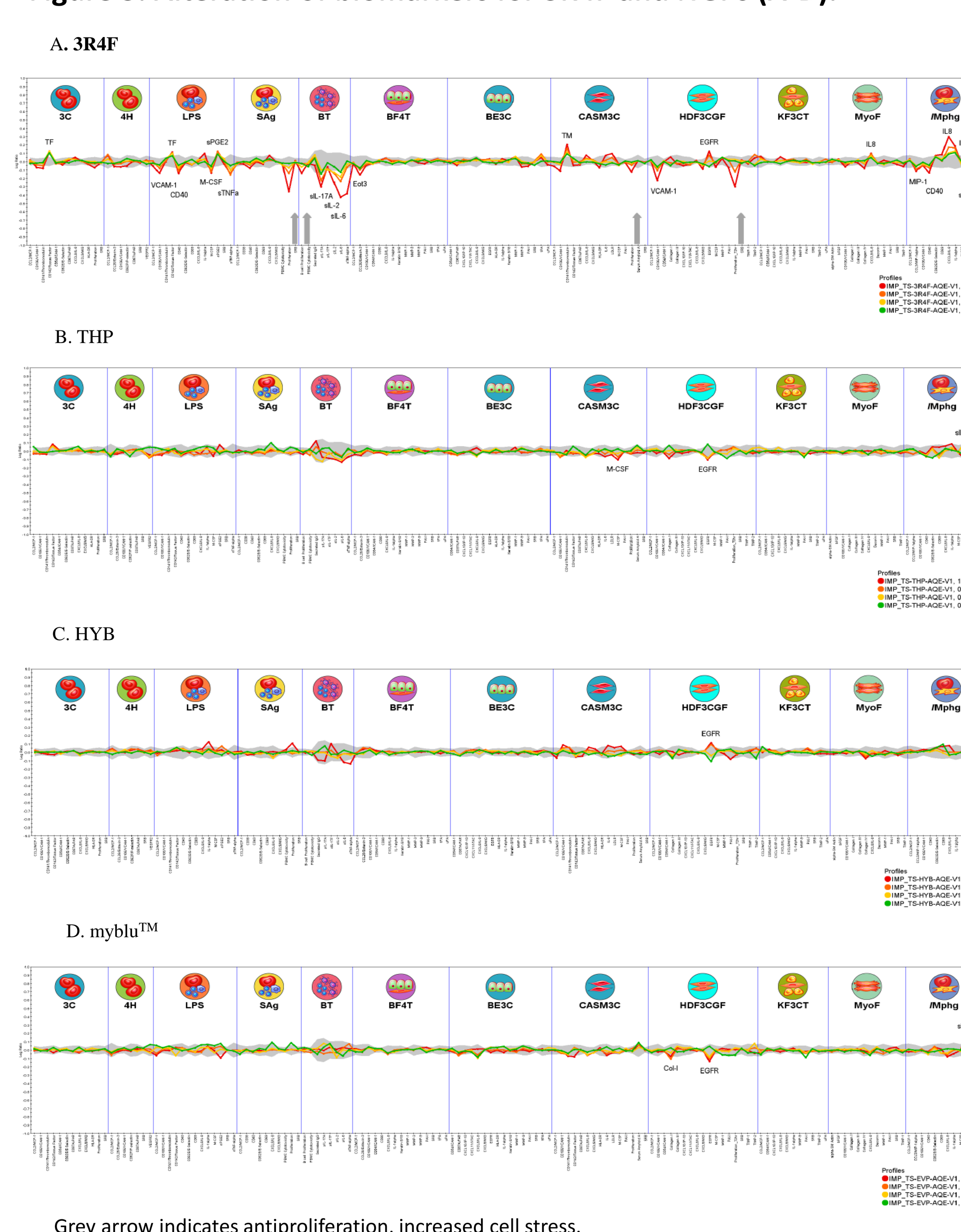
Table 3: Summary of nicotine carbonyls trapped in PBS samples

Concentration µg/ml	3R4F	HTP	HYP	myblu™	LOQ (µg/ml)
Nicotine	82.5	123.0	53.0	152.0	0.01
Formaldehyde	5.9	0.9	1.0	<LOQ	0.25
Acetaldehyde	157.1	52.9	<LOQ	<LOQ	1.5
Acetone	24.0	5.4	<LOQ	<LOQ	1.0
Acrolein	9.4	1.3	0.5	<LOQ	0.5
Propionaldehyde	9.5	3.5	<LOQ	<LOQ	0.5
Crotonaldehyde	6.2	0.6	<LOQ	<LOQ	0.5
2-Butanone (MEK)	6.3	1.3	<LOQ	<LOQ	0.5
n-Butyraldehyde	3.6	2.8	<LOQ	<LOQ	0.5

LOQ: Limit of quantification

As expected, there were significant levels of carbonyls trapped in PBS for 3R4F. Fewer carbonyls were present for THP and there were minimal to carbonyls below LOQ trapped for HYB and EVP.

Figure 3: Alteration of biomarkers for 3R4F and NGPs (A-D).



Grey arrow indicates antiproliferation, increased cell stress.

For the BioMAP profiles there were significant alterations in biomarkers seen for the 3R4F sample when compared to those seen for the three NGPs. The three NGPs exhibited minimal changes at the maximum concentration of 1% PBS.

Table 4: List of altered Biomarkers per device

Biological and disease relevance activity	Decreased biomarker activity	Increased biomarker activity	Modulated biomarker activity
3R4F			
Inflammation-related activities	MIP-1, TNFa, VCAM-1, Eot3	sPGE2, IL-1a, IL-8	_
Immunomodulatory activities	M-CSF, sIL-10, sIL-2, sIL-6, CD40, sIL-17A		_
Tissue remodelling activities		EGFR	_
Haemostasis-related activities		TM, TF	_
HTP			
Immunomodulatory activities	M-CSF	sIL-10	_
Tissue remodelling activities	EGFR		_
HYB			
Tissue remodelling activities		EGFR	_
myblu™			
Immunomodulatory activities		sIL-10	_
Tissue remodelling activities	EGFR, Col-I		_

Significant biomarker alterations were observed for 3R4F across multiple cell systems at concentrations from 0.25%. For THP, HYB and EVP, biomarker alterations were three or less and very close to the control envelope, or seen in isolated cell systems. There was no antiproliferation when tested up to 1% in PBS for the NGPs.

The biomarker traces were then further analysed for toxicity profiles of known mechanisms of action using the BioMAP Toxicity Signatures Analysis, results are summarised in Table 5.

Table 5: Summary of Toxicity Signatures

Test Agent	Potential Toxicity Detected								
	Acute Toxicity	Immuno-Suppression	Skin Irritation	Liver Toxicity	Organ Toxicity	Skin Rash (MEK-related)	Skin Sensitization	Thrombosis	Vascular Toxicity
IMP_TS-3R4F-AQE-V1	nd	>= 0.25 %	>= 0.25 %	nd	nd	nd	nd	>= 0.12 %	nd
IMP_TS-THP-AQE-V1	nd	nd	nd	nd	nd	nd	nd	nd	nd
IMP_TS-HYB-AQE-V1	nd	nd	nd	nd	nd	nd	nd	nd	nd
IMP_TS-EVP-AQE-V1	nd	nd	nd	nd	nd	nd	nd	nd	nd

For 3R4F there was a signature detected for thrombosis at the lowest dose tested (0.12%) and significant signatures for immunosuppression and skin irritation at 0.25%. None of the NGPs showed toxicity signatures. The broad mechanisms of action identified for 3R4F by Toxicity Signature Analysis are **immunosuppression**: depressed responses of immune cells; **skin irritation**: sustained production of prostaglandins leading to vascular permeability; **thrombosis:** leukocyte infiltration and promotion of Th17 responses and modulators of vascular autophagy leading to increased thrombotic potential within the vasculature. Smoking has previously been reportedly linked to these end points in the scientific literature (Arnson *et al.*, (2010); Sorenson *et al.*, (2010); Tapson (2005)).

4. CONCLUSIONS

- There was a significant alteration of multiple biomarkers following 3R4F exposure. In contrast to fewer or no reproducible alterations meeting significance criteria for the NGPs at the top concentration of 1% PBS under the experimental test conditions.
- Toxicity signatures were observed for 3R4F at concentrations of 0.12% (thrombosis) and/or 0.25% (immunosuppression and skin irritation). Toxicity signatures were not observed for any of the NGPs up to 1%, under the experimental test conditions.
- This assay adds to the weight of evidence of potentially reduced toxicity of NGPs when compared to conventional cigarettes

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