E-cigarettes induce lower biological responses than conventional cigarettes: A comparison of *in vitro* toxicity following repeated whole aerosol exposure to human bronchial tissue for 4 weeks

Lukasz Czekala[1], Roman Wieczorek[2], Edgar Trelles Sticken[2], Lisa Maria Bode[2] Liam Simms[1],Matthew Stevenson[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

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1. INTRODUCTION

Smoking is a cause of serious diseases in smokers, including lung cancer, heart disease and emphysema. There is scientific agreement that the harmful toxicants formed during tobacco combustion are the cause of smoking-related diseases, not nicotine. For this reason numerous public health bodies and governments worldwide have indicated that e-cigarettes have a central role to play in tobacco harm reduction. Previously, we have shown that acute exposures of e-cigarette aerosol to 3D tissue resulted in no significant toxicity compared to matched air controls (Czekala *et al.*, 2019). In this study we compared the *in vitro* toxicological responses of a 3D organotypic model of the human airway epithelia (MucilAir™, Epithelix) following repeated exposures to either myblu™ whole undiluted aerosol or Kentucky Reference Cigarette (3R4F) smoke (1:17 dilution) in a range of functional endpoints.

2. MATERIALS AND METHODS

2.0 Test Articles

- Kentucky 3R4F Reference Cigarette
- *my*blu™ device *(Figure 1)* and pod (1.6% [w/w] nicotine; tobacco flavour), UK market

2.1 Tissues

MucilAir™ tissues, a fully differentiated 3D airway epithelium, were purchased from Epithelix Sàrl and cultured for more than 7 days before use. The tissues were exposed to test smoke/aerosol under the conditions listed in 2.2.

2.2 Smoke and Aerosol Generation

Test product aerosol/smoke was generated according to *Table 1*. Tissues were repeatedly exposed (3 times per week) at the Air Liquid Interface (ALI) for 4 weeks to either 30, 60 or 90 puffs of aerosol/smoke/filtered humidified air control using Imperial Brands' Smoke Aerosol Exposure In Vitro System (SAEIVS) (Burghart Tabaktechnik, Wedel, Germany). Cigarette smoke was diluted with filtered humidified air 1:17 times whilst myblu™ aerosol was applied undiluted. Following each individual exposure, tissues were incubated for 24 hours at standard culture conditions before further repeated exposures.

Test product	Smoking regime	Puff volume (ml)	Puff duration (s)	Puff interval (s)	Ventilation blocking	Puff profile	Smoking machine
3R4F	Health Canada Intense [2]	55	2	30	Yes	Bell	SAEIVS / linear like
myblu™	Coresta Recommended Method N°81 [3]	55	3	30	N/A	Square	SAEIVS / linear like

Table 1: Smoke and Aerosol Generation regimes

2.3 Dosimetry

The nicotine dose delivered to the 24 Multi-Well Plate (MWP) filled with Phosphate-buffered saline (PBS) was analysed using an AB Sciex API 6500 (Q-Trap) with an Agilent (1290 Infinity) HPLC system to ensure smoke/vapour delivery to the model. The Electrospray Ionization (ESI) and multiple reaction monitoring in positive ion mode was used. The quantification was performed with an external calibration of nicotine with a range from 2.5-100 ng/ml.

2.4 Tissue viability and barrier integrity

Cellular cytotoxicity was assessed via the LDH quantification in growth culture media at each exposure time (data not shown). Barrier integrity of each tissue was assessed weekly by measuring the Transepithelial Electrical Resistance (TEER) using an EVOM2 Epithelial Voltohmmeter. Morphological changes were observed by microscope and documented.

2.5 Histology and immunofluorescence staining

Tissue architecture was assessed using H&E/Alcian Blue staining. Fox-J1 and MUC-5-AC (data not shown) immunostaining were used to stain for ciliated cells and mucin, respectively. IL-13 served as a positive control for goblet cell hyperplasia.

2.6 Inflammatory markers

Inflammatory markers (IL-1β; IL-6; IL-8; TNF-α; MMP-1, MMP-3 & MMP-9) secreted into the culture media were collected from each well prior the exposure on the exposure day and were frozen at -80°C for further analysis. Assessment of cytokines, chemokines and matrix metalloproteinases were performed with MESO Scale QuickPlex™ on custom made plates according to the manufacturers recommendations (MESO Scale).

2.7 Cilia Beat Frequency and Active Area

Cilia activity were measured every week on Tuesday and Friday, 4 hours after exposure to each agent/control. Cilia Beat Frequency (CBF) and Active Area (CAA) were evaluated under 4x magnification using SAVA software.

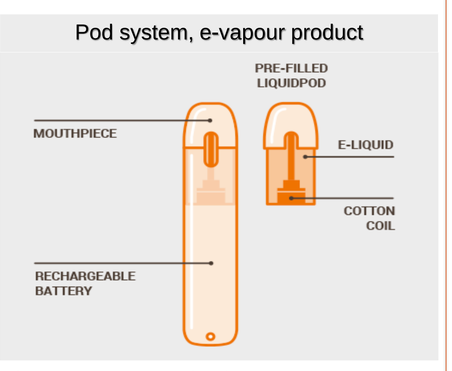


Figure 1: *E-Vapour product format*

2.8 Data and statistical analysis

All data and statistical analysis were conducted using Microsoft Excel and GraphPad Prism. Statistically significant differences between samples were calculated using ANOVA with posthoc Dunnett's test. All differences were considered statistically significant with a p-value ≤ 0.05.

3. RESULTS

3.1 Dosimetry of Smoke / Aerosol

The nicotine delivered to 24 MWP plates was quantified and results are presented in *Figure 2*. Nicotine delivery for the 3R4F is lower than corresponding myblu™ (undiluted) due to the 1/17 dilution of the smoke. A good dose correlation is observed.

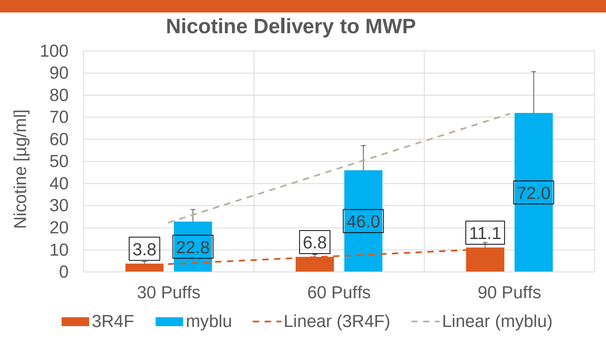


Figure 2: *Nicotine delivery to 24 MWP*

3.2 *In Vitro* Toxicology

3.2.1 Tissue Viability and Barrier Integrity

The TEER value decreased significantly after exposure to the 3R4F smoke. This suggest a major loss of model barrier integrity. No differences in TEER were observed after exposure to the myblu™ aerosol in comparison with matched air control.

LDH levels (data not shown) did not distinguish between exposure groups during the study, except 90 puffs of 3R4F.

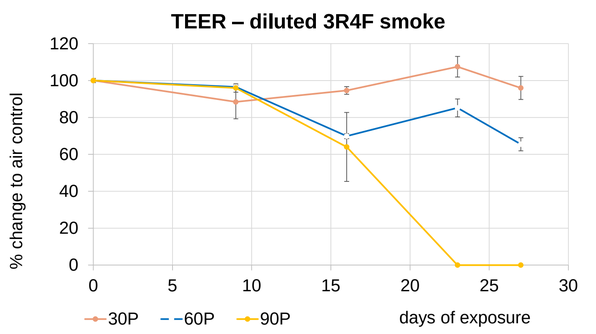


Figure 3: *Change of TEER relative to air control*

3.2.2 Histology and immunofluorescence staining

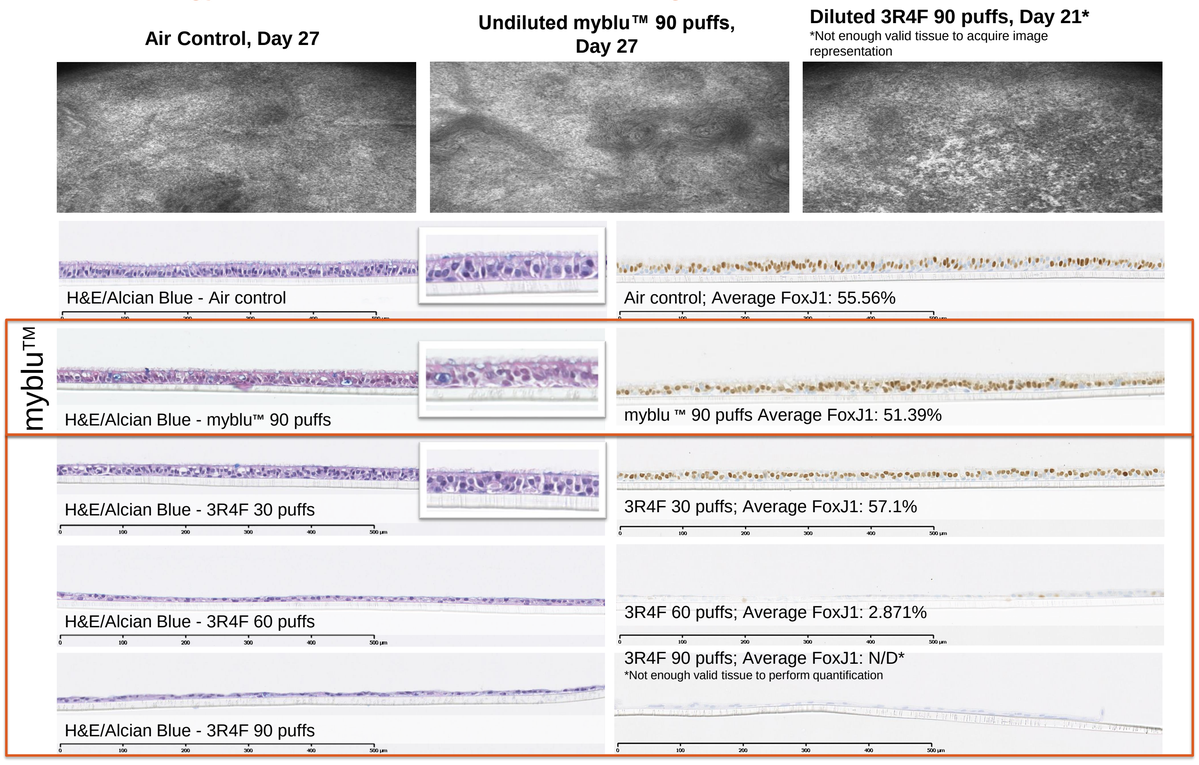


Figure 4: *Histological evaluation (H&E/Alcian Blue and FoxJ1) after 4 weeks of repeated exposure to 30,60 and 90 puffs of undiluted myblu™ aerosol and 3R4F cigarette smoke diluted 1/17. Air control consists of 90 puffs of humidified filtered air.*

After 4 weeks of repeated exposure to myblu™ aerosol tissues, were indistinguishable from matched air control for both H&E/Alcian Blue and FoxJ1. There was no decrease in cilia number (visual inspection of H&E slides) nor to the number of ciliated cells (expressed as % of FoxJ1). A dose dependant decrease in cilia and ciliated cells was observed after exposure to 3R4F cigarette smoke. For 60 and 90 puff doses no cilia/ciliated cells remained at the end of the experiment. Dose dependent cell loss was observed; the 3R4F 90 puff dose was terminated on day 21 due to excessive cell loss. Histological evaluation indicates only few basal cells remaining in the model. Except for IL-13, no increase of goblet cells was detected during the study after MUC-5-AC staining (data not shown).

3.2.3 Inflammatory markers assessment

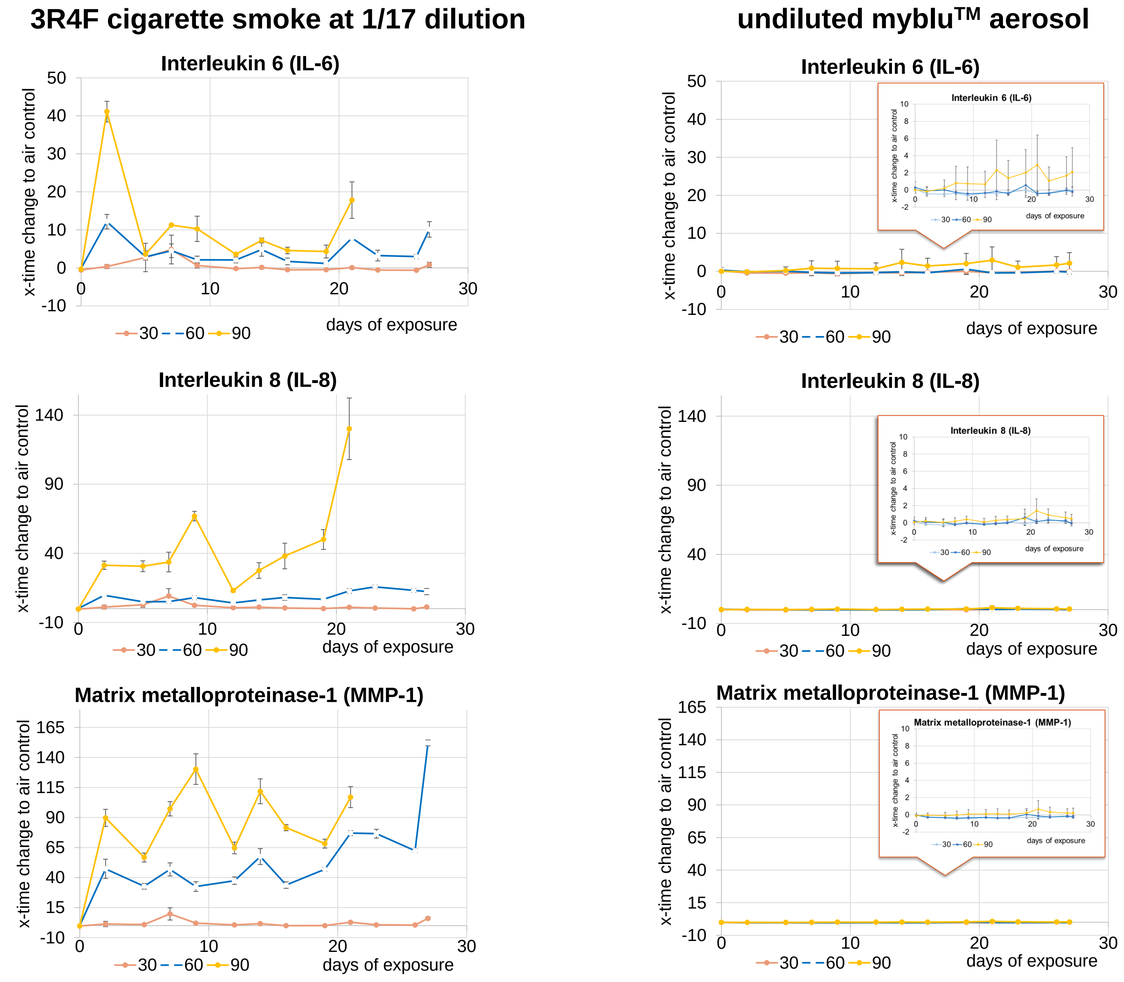
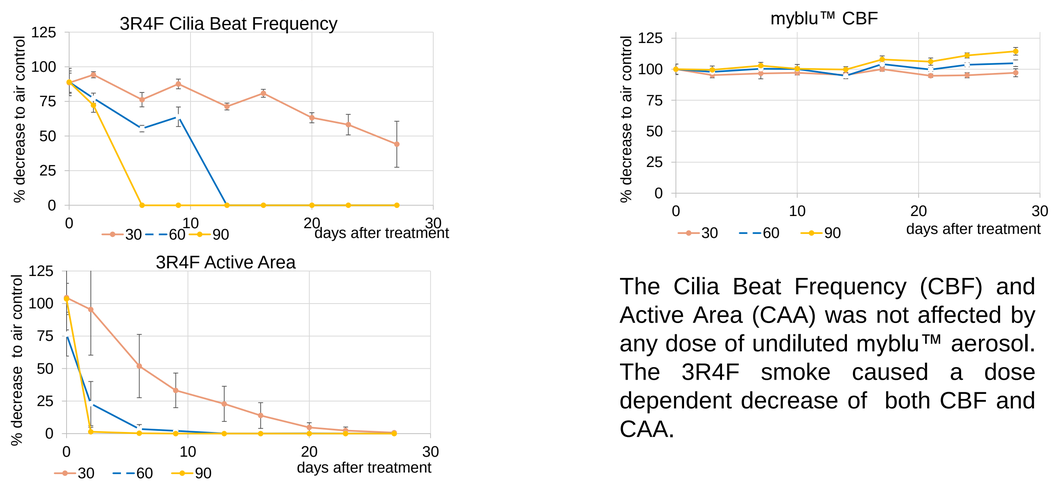


Figure 5: *Cytokines IL-6, IL-8 and matrix metalloproteinase 1 (MMP-1) level changes during 4 week (21 Days for 90 puffs of 3R4F) repeated exposure to whole aerosol/smoke/humidified air assessed in culture media.*

The undiluted myblu™ aerosol at any dose tested did not alter cytokine secretion (IL-8, IL-6, MMP-1 – data shown, and IL-1β, TNF-α, MMP-3, MMP-9 – data not shown) in comparison to match air control.
Despite smoke dilution, the 3R4F cigarette significantly altered the secretion of all cytokines mentioned above at 60 and 90 puff dose (representative cytokines presented).

3.2.4 Cilia Beat Frequency



The Cilia Beat Frequency (CBF) and Active Area (CAA) was not affected by any dose of undiluted myblu™ aerosol. The 3R4F smoke caused a dose dependent decrease of both CBF and CAA.

Figure 6: *Cilia Beat Frequency and Cilia Active Area assessment expressed as a % decrease to matched air control.*

4. CONCLUSIONS

- Although myblu™ delivered significantly more nicotine compared to the 3R4F cigarette smoke, it did not trigger any significant toxicological response in any of the models compared with matched air controls.
- Data demonstrates a dose response to diluted (1/17) cigarette smoke in various endpoints assessed, including changes to tissue morphology at 30 (loss of cilia) 60 and 90 puffs, with significant increases in selected cytokines (IL-1β, IL-6, IL-8, MMP-1, MMP-3, MMP-9 and TNF-α).
- Where cigarette smoke significantly decreased CBF, CAA and number of cilia and ciliated cells at all doses tested, tissues exposed to myblu™ aerosols were indistinguishable from matched air control.
- In overall, these results add to a weight-of-evidence approach to substantiate the harm reduction potential of myblu™ for adult smokers.

REFERENCES

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