



1. Introduction and Objectives

Smoking is a cause of serious disease in smokers. Tobacco-based and tobacco-free next generation products (NGPs) are believed to be a less harmful alternative to cigarettes, thereby creating a huge global public health opportunity if significant numbers of adult smokers fully switch. Due to the evolving regulatory landscape and dynamic nature of innovation of NGPs, new assays are required to quickly determine the potential biological impact of these products on adult smokers. High Content Screening (HCS) is part of a wider set of *in vitro* pre-clinical assays that can be used to assess NGP aerosol for any potential toxicity together with clinical studies adding to the overall weight of evidence in product assessment. The HCS enables the detection of early cellular events in human primary cell lines in response to test materials. Previous work has assessed the impact of e-liquid formulations on a variety of cellular endpoints using HCS on normal human primary lung cells (NHBEs) (Czekala *et al.*, 2019). This study showed that 3R4F TPM was the most biologically active test compound, inducing most of the cellular end points at concentrations, typically one hundred times lower than that for all the e-liquids. As a follow up we investigated the impact of exposing NHBEs to bubbled aerosol extracts obtained from a range of tobacco and non-tobacco based NGPs and compared it to the impact of cigarette smoke in a range of endpoints in an HCS approach. Bubbling of Phosphate buffered saline (PBS) with NGP aerosol and cigarette smoke has been used by others to deliver compounds to the cellular system that are human physiologically relevant (Kogel *et al.*, 2015).

2. Materials and Methods

2.1 Cell culture

Human normal bronchial epithelial cells from a single donor (60/ male/ Caucasian/ non-smoker) were obtained from Promocell (Order number: C-12640) and maintained at 37°C in an atmosphere of 5% CO₂ in Airway Epithelial Cell Growth Medium (AEGM). AEGM consisted of AEGM (Promocell, C-21060) complemented with SupplementMix (Promocell, C-39165) containing Bovine Pituitary Extract 0.004ml/ml, epidermal growth factor (10ng/mL), insulin (5µg/mL), Hydrocortisone (0.5µg/mL), Epinephrine (0.5µg/mL), Triiodo-L-thyronine (6.7 ng/mL); Transferrin, holo (10µg/mL), and Retinoic acid (0.1ng/mL). Sub cultivation was performed with a cell seeding density of 2.5E6 cells per flask (T175). Experiments were conducted with cells from 5th to 12th passage only. Thereafter cell growth potential decreased rapidly due to the cellular Hayflick-limit.

2.2 Test Samples

3R4F Kentucky Reference Cigarettes (3R4F), Tobacco Heated Product (THP), Hybrid Product (HYB): 1.8 % nicotine, mybluTM Tobacco flavour: 1.6% nicotine (myblu)

2.3 Smoke / Aerosol Extract Generation Method

Aerosol from test products was generated with a Vitrocell VC10s (Vitrocell, Waldkirch, Germany) smoking machine (See Table 1 for aerosol generation regimens). Smoke / aerosol extracts were prepared by bubbling the sample aerosol into 3 in-line impingers each containing 10 mL PBS (see Figure 1). The bubbled PBS (bPBS) solution from the single impingers were pooled (total of 30 mL) and used to prepare the exposure dilutions (max dosing of 4% from 3R4F smoke and 10% from the other platforms).

Table 1: Aerosol was generated for products using the following regimens:

Sample	Smoking Regime	Puff Volume (ml)	Puff Duration (Sec)	Puff Interval (Sec)	Vent Blockin g	Puff Profile	Resulting concentration [puff/ml]; nicotine [µg./ml]
3R4F Cigarette	Health Canada Intense	55	2	30	Yes	Bell Shaped	1.8/mL 86 ± 12µg/mL
THP	Health Canada Intense	55	2	30	N/A	Bell Shaped	4.0/mL; 150 ± 17µg/mL
HYB	Coresta Recommended Method 81	55	3	30	N/A	Square Wave	4.0/mL; 70 ± 1µg/mL
myblu TM	Coresta Recommended Method 81	55	3	30	N/A	Square Wave	4.0/mL; 175 ± 17µg/mL



Figure 1: row of 3 impingers to trap the different aerosols

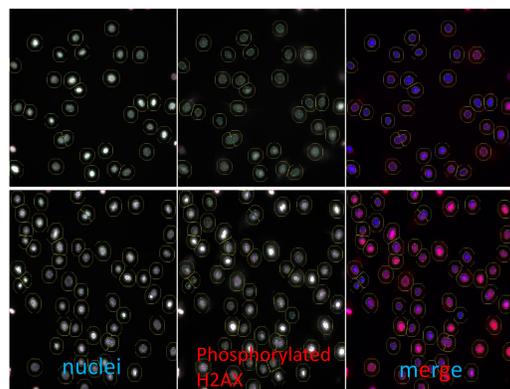


Figure 2: Representative images from immunostaining against phosphorylated histone H2AX following 24h incubation with negative control (10% PBS, upper row) and 24h incubation of cells with 4% 3R4F bPBS (lower row). Staining patterns indicate an overlap of different mechanisms that include phosphorylation of histone H2AX (DNA damage, apoptosis, senescence, S-phase defects).

2.4 Antibodies and kits used

Primary antibodies used were: 1. Mouse-anti phospho-histone H2AX (Ser139 clone JBW301Cat. # 05-636 Millipore), Rabbit anti-NF-kB (abcam ab32536), Mouse-anti-Cytochrome C Antibody (Abcam ab110325), Rabbit anti-c-Jun (phospho S36; Abcam ab32385), Thioltracker (Thermo). Cells were counter stained with 4',6-Diamidin-2-phenylindol (DAPI). Following the exposure period cells were fixed with 4% formaldehyde in PBS, permeabilized with 0.1% TritonTM X-100 in PBS, and blocked with 3% BSA in PBS for an hour. Primary antibodies were applied with 1:500 dilutions in antibody solution (PBS / 0.1% Tween 20/ BSA) and incubated over night at 4°C. On the next day primary antibodies were detected with appropriate secondary antibodies. For the determination of the cellular glutathione (GSH) content a Thioltracker kit (Thermo; T10095) was used according to the supplier's instructions.

2.5 Evaluation

The dose responses were calculated as fold changes compared to the negative control values (corresponds to 1 on the y-axis). The normalised results for each marker from the different test days were averaged and statistically analysed in GraphPad Prism 8.2.0. Statistical significance was determined using one sided ANOVA with posthoc-Dunnett test. Dose response was evaluated with linear trend analysis. A response was regarded as relevant when a reproducible 20% difference and statistical significance as compared to the negative control was reached (i.e. 1.2 or 0.8 fold as compared to negative control). Results are all displayed on % bPBS basis.

3. Results

3.1 Osmolarity of solutions

The different dilutions of bPBS in medium did not show relevant increases in osmolarity with values ranging between 280 and 310 mOsm. The values indicate that no secondary artefacts due to osmotic stress had to be expected.

3.2 Biological effects

NHBE cells were treated for 4 and 24 hours before subsequent antibody staining. Determination of GSH depletion was performed following a 2-hour exposure to avoid the influence of an adaptive response of the cells. Only the health markers with significant effects are shown. No effect for Cytochrome C release could be observed when treated with the different extracts (data not shown).

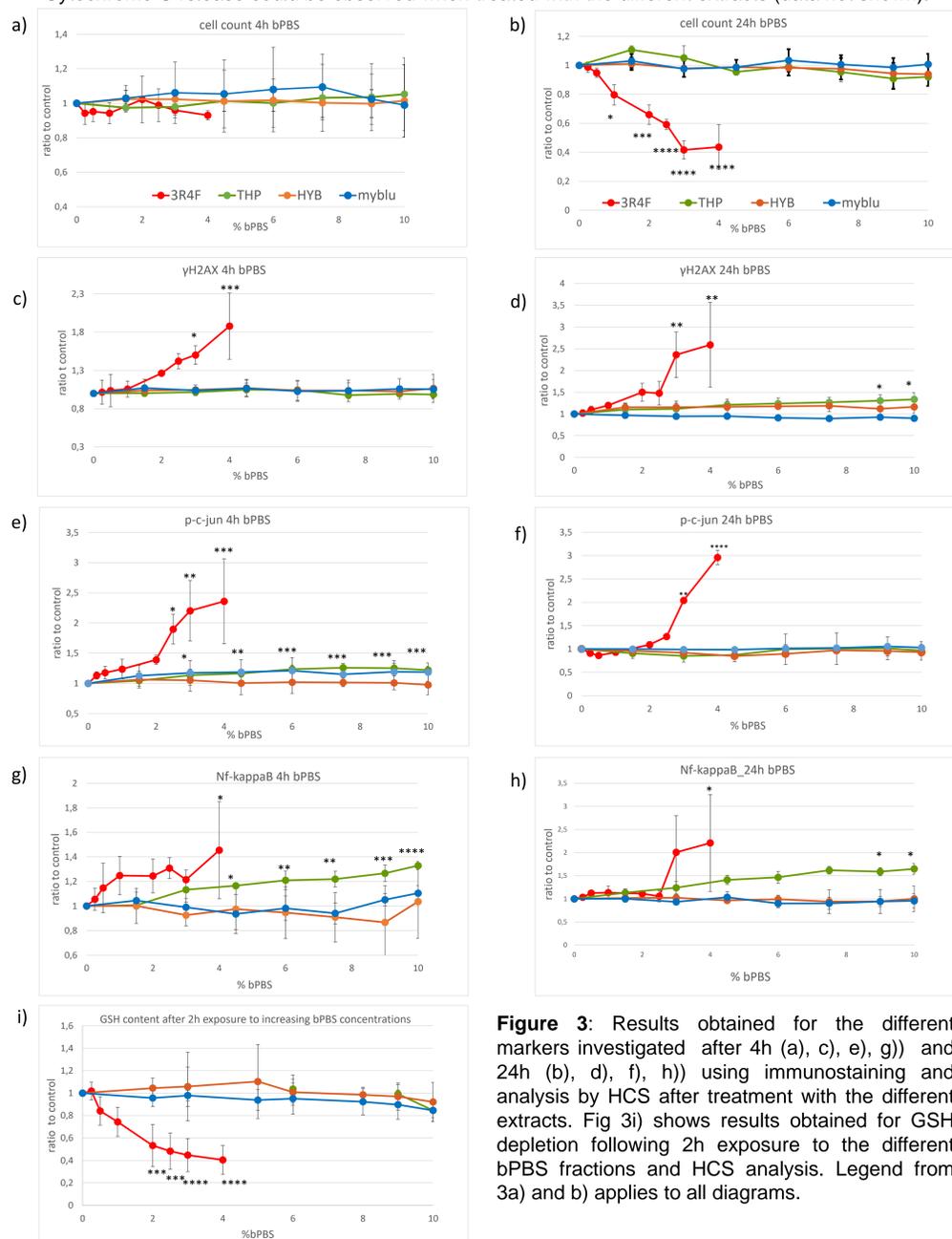


Figure 3: Results obtained for the different markers investigated after 4h (a, c, e, g)) and 24h (b, d, f, h)) using immunostaining and analysis by HCS after treatment with the different extracts. Fig 3i) shows results obtained for GSH depletion following 2h exposure to the different bPBS fractions and HCS analysis. Legend from 3a) and b) applies to all diagrams.

- These results suggested that low concentrations of 3R4F bPBS trigger different endpoints in the cell (oxidative stress, translocation of Nf-kappaB, phosphorylation of c-jun, and phosphorylation of histone H2AX) that resulted in statistically significantly reduced cell counts following 24h of exposure.
- No statistically significant effects were observed with up to 10% bPBS from HYB and myblu (corresponding to 7µg nicotine /mL and 18µg nicotine /mL respectively).
- Cells treated with the THP bPBS showed increased phosphorylation of c-jun and translocation of NfκB as well as phosphorylation of histone H2AX following 4h and 24h treatment respectively (see Figure 3 d,g,e,h).

4. Conclusions

- Comparing the results obtained under the conditions of this study the ranking with regard to harm potential of the different platforms on NHBEs can be defined as 3R4F>THP>HYB=myblu.
- THP was able to induce some endpoints though to a lower extent and at higher bPBS concentrations than 3R4F.
- The data indicate that myblu and HYB did not induce any of the endpoints investigated even with bPBS concentrations that correspond to five-fold increased nicotine levels (for myblu) when compared to 3R4F.
- The present results support a weight of evidence approach to substantiate the reduced harm potential of e-vapor products.