Next generation product aerosol bubbled extracts show little to no effect in high content screening endpoints when compared to cigarette smoke bubbled extracts

1. INTRODUCTION and Objectives

Smoking is a serious cause of 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 preclinical assays that can be used to assess NGP aerosol for any potential toxicity. This study showed that 3R4F TPM was the most biologically active test compound, inducing most of the cellular endpoints at concentrations, typically one hundred times lower than that for all the 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 highly physiological 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) containing Bovine Fetal Calf Serum (0.04%/ml), epidermal growth factor (10%/ml), insulin (5/µg/ml), Hydrcortisone (0.5/µg/ml), Epinephrine (0.5/µg/ml), Triolco-L Thyroline (6.7 ng/ml), Transferin, hbo3 (10/µg/ml), and Retinoic Acid (0.1/µg/ml). Subcultivation was performed with a cell seeding density of 2.5×10⁶ 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, myblu™ Tobacco flavour: 1.6% nicotine (myblu)

2.3 Smoke / Aerosol Extract Generation Method

Aerosol from test products was generated with a Vitrocel VC110s (Vitrocel, 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 15 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 dumb platform).

2.4 Antibodies and kits used

Primary antibodies used were: 1. Mouse-anti-phospho-histone H2AX (Ser139 clone JBW301/2, Abcam # 05-636 Millipore), Rabbit anti-NF-kB (abcam ab23536), Mouse-anti-Cytodrome C Antibody (Abcam ab110225), Rabbit anti-c-jun (phospho s363; Abcam ab23385), Thiobracer (Thermo). Cells were counter stained with 4:6-Diamidino-2-phenylindol (DAPI). Following the exposure period cells were fixed with 4% formaldehyde in PBS, permeabilized with 0.1% Triton 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 overnight 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 Thiohirat 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 days were averaged and statistically analysed in GraphPad Prism 8.2.0. Statistical significance was determined using one sided ANOVA with posthoc Dunnet 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.

2.6 Statistical analysis

All results were log transformed for statistical 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.

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Smoking Regimen</th>
<th>Pulldown Volume</th>
<th>Pulldown Duration</th>
<th>Pulldown Incubation</th>
<th>Bubbling</th>
<th>Pulldown Profile</th>
<th>Concentration (µg/mL)</th>
<th>NHBE</th>
<th>Confluent (100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3R4F Cigarette</td>
<td>Health Canada Intense</td>
<td>55</td>
<td>2</td>
<td>30</td>
<td>Yes</td>
<td>Bell</td>
<td>Shaped</td>
<td>BPS</td>
<td>86.4±15µg/mL</td>
</tr>
<tr>
<td>TIP</td>
<td>Health Canada Intense</td>
<td>55</td>
<td>2</td>
<td>30</td>
<td>N/A</td>
<td>Bell</td>
<td>Shaped</td>
<td>THP</td>
<td>4.0µg/mL</td>
</tr>
<tr>
<td>HYB</td>
<td>Recommended Method B1</td>
<td>55</td>
<td>3</td>
<td>30</td>
<td>N/A</td>
<td>Square</td>
<td>Wave</td>
<td>MYBLU</td>
<td>4.0µg/mL</td>
</tr>
<tr>
<td>myblu™</td>
<td>Recommended Method B1</td>
<td>55</td>
<td>3</td>
<td>30</td>
<td>N/A</td>
<td>Square</td>
<td>Wave</td>
<td>THP</td>
<td>4.0µg/mL</td>
</tr>
</tbody>
</table>

3. Results

3.1 Osmolarity of solutions

The different dilutions of bPBS in medium did not show relevant changes 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 GHSM depletion was performed after 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).

4. Conclusions

- These results suggested that low concentrations of 3R4F-TPPs 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 7ng nicotine /mL and 18µg nicotine /mL respectively).
- Cells treated with the THP bPBS showed increased phosphorylation of c-jun and translocation of NIKb as well as phosphorylation of histone H2AX following 4h and 24h treatment respectively (see Figure 3 d-g,h).

5. References

1. Czekala et al., (2019) High Content Screening in NHBE cells shows significantly reduced biological activity of flavoured e-liquids, when compared to cigarette smoke condensate. Toxicol In Vitro; 58:86-96

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1. Czekala et al., (2019) High Content Screening in NHBE cells shows significantly reduced biological activity of flavoured e-liquids, when compared to cigarette smoke condensate. Toxicol In Vitro; 58:86-96