# The use of High Content Screening (HCS) in human primary lung cells to assess e-liquids

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## 1. INTRODUCTION

Due to the evolving regulatory landscape and dynamic nature of innovation with e-cigarettes, new assays are required to quickly determine the subtle biological response of e-liquids for stewardship purposes. The HCS assay enables the detection of early cellular events in human primary cell lines in response to test materials. The aim of this study was to assess the cellular effects of commercial e-liquids using High Content Screening (HCS) on human primary lung cells. Additionally, cellular effects were compared to a reference cigarette smoke condensate and flavourless e-liquids containing different concentrations of nicotine.

## 2. MATERIALS & METHODS

### Test Materials

Three experimental e-liquids: Base Liquid (PG/VG: 50/50); Base Liquid + 1.2% nicotine (PG/VG/Nic: 49.4/49.4/1.2); and Base Liquid + 2.4% nicotine (PG/VG/Nic: 48.8/48.8/2.4). Four commercially available e-liquids, with varying flavours all at 2.4% nicotine (W/W) strength, US formulations. Cigarette Smoke Condensate (CSC) was collected using Intense smoking regime on Borwaldt RM-20 smoking machine at a target smoke total particulate matter (TPM) of 50 mg/ml of DMSO.

### Methodology

Normal Human Bronchial Epithelial (NHBE) cells (Supplied by Lonza, Cologne, Germany) were cultured in 96-well plates, at a seeding density of $9x10^3$ cells per well, followed by a 24-hour incubation at 37˚C and 5% $CO_2$. After the incubation period the cells were exposed to the test materials for 24 hours. The cellular responses of e-liquid were compared to cells exposed to 3R4F Reference Cigarette Smoke Condensate (CSC). High Content Imaging was performed using an automated fluorescent cellular imager (Cellomics® ArrayScan VTI). A minimum of eight individual images were acquired per fluorescent channel for each well of the experimental plates. All experimental work was conducted by Cyprotex laboratories, UK. Osmotic concentrations were measured on a Osmomat 030 (Gonotec GmbH) osmometer. Due to the osmotic nature of e-liquids themselves, cells were exposed to a maximum concentration of 3% for 24 hours (based on previous studies and scientific literature[1]).

## 3. RESULTS

**Table 1:** Minimum Effective Concentration (MEC) for specific cellular endpoints in response to 3R4F CSC and e-liquids

| | High Content Screening - Biological Response Heat Map | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3R4F CSC (0.001 – 0.02%) | | BL 0% (0.5, 1.0, 3%) | | BL 1.2% (0.0313 – 3%) | | BL 2.4% (0.0313 – 3%) | | Blueberry 2.4% (0.0313 – 3%) | | Tobacco 2.4% (0.0313 – 3%) | | Menthol 2.4% (0.0313 – 3%) | | Vanilla 2.4% (0.0313 – 3%) | |
| | MEC | AC50% | MEC | AC50% | MEC | AC50 | MEC | AC50 | MEC | AC50 | MEC | AC50 | MEC | AC50 | MEC | AC50 |
| Cell count | ↓0.001 | 0.008 | NR | NR | NR | NR | ↓2.1 | >3ǂ | ↓1.06 | 1.78 | ↓1.36 | 2.08 | ↓0.36 | 0.885 | ↓1.48 | 1.89 |
| Nuclear size | NR* | NR* | NR | NR | ↓0.886 (NS) | >3ǂ (NS) | ↓0.031 (NS) | >3ǂ (NS) | ↓0.17 | 2.55 | NR | NR | ↓0.212 | 1.79 | ↓0.0708 (NS) | >3ǂ (NS) |
| DNA Structure | NR* | NR* | NR | NR | NR | NR | NR | NR | NR | NR | ↓0.546 | >1ǂ | NR | NR | NR | NR |
| Cell Cycle Arrest | ↑0.001 | >0.005 | NR | NR | ↑1.69 | 2.0 | ↑0.718 | 0.982 | ↑0.377 | 0.598 | ↑0.363 | 0.548 | ↑0.0551 | 0.096 | ↑0.406 | 0.573 |
| Cell Membrane Permeability | ↑0.001 (NS) | 0.02ǂ (NS) | NR | NR | ↑0.345 (NS) | >3ǂ (NS) | ↑0.149 (NS) | >3ǂ | ↑0.783 | 1.22 | ↑1.52 | 2.05 | ↑0.659 | 0.855 | ↑1.64 | 2.06 |
| Caspase 3/7 Intensity | ↑0.001 | >0.015ǂ | NR | NR | NR | NR | NR | NR | ↑0.596 | >3ǂ | ↑0.652 | >3ǂ | ↑0.106 | >2ǂ | ↑1.17 | >3ǂ |
| NF-κB | ↑0.005 | >0.02ǂ (NS) | NR | NR | NR | NR | NR | NR | ↑1.12 | 1.86 | ↑1.86 | 2.54 | ↑0.63 | 0.991 | ↑1.9 | 2.42 |
| Mitochondrial Mass | ↑0.006 | >0.02ǂ | NR | NR | NR | NR | ↓0.8 (NS) | >3ǂ (NS) | NR | NR | ↑1.88 | >3ǂ | ↑0.88 | >1ǂ | ↑2.98 | >3ǂ |
| Mitochondrial Membrane Potential (Δψm) | ↓0.005 | 0.012 | NR | NR | NR | NR | NR | NR | ↓0.865 | 1.05 | ↓0.88 | 1.94 | ↓0.689 | 1.36 | ↓1.16 | 2.32 |
| Oxidative Stress | ↓0.005 | 0.009 | NR | NR | NR | NR | NR | NR | NR | NR | ↓0.836 | 1.61 | ↓0.833 | 1.63 | ↓1.08 | 1.96 |
| Glutathione Content | ↓0.008 | 0.008 | NR | NR | NR | NR | NR | NR | ↓1.35 | 1.65 | ↓1.77 | 2.14 | ↓0.735 | 0.816 | ↓1.9 | 2.15 |
| Cellular ATP | ↑0.006 | >0.02ǂ | ↓2.59 | >3 | ↓1.55 (NS) | >3ǂ (NS) | ↓1.02 | 1.82 | ↓0.952 | 1.31 | ↓1.76 | 1.76 | ↓0.602 | 0.773 | ↓1.62 | 1.71 |

MEC = Minimum Effective Concentration that significantly crosses vehicle control threshold (red dotted lines Figure 1) . NR = no response observed. NR* = no response observed, possibly due to the high sample cytotoxicity. $AC_{50}$ = the concentration at which 50% maximum effect is observed for each cell health parameters. ǂ = $AC_{50}$ was calculated, but is greater than the maximum surviving concentration. Arrows indicate increase or decrease for specific cellular endpoint. BL = Base liquid 50:50 PG/VG with nicotine %. 3R4F CSC was tested between 0.001-0.02% and the e-liquids between 0.0313-3%.

**Figure 1:** Changes in cellular glutathione content in response to 3R4F CSC & various e-liquids after 24h exposure

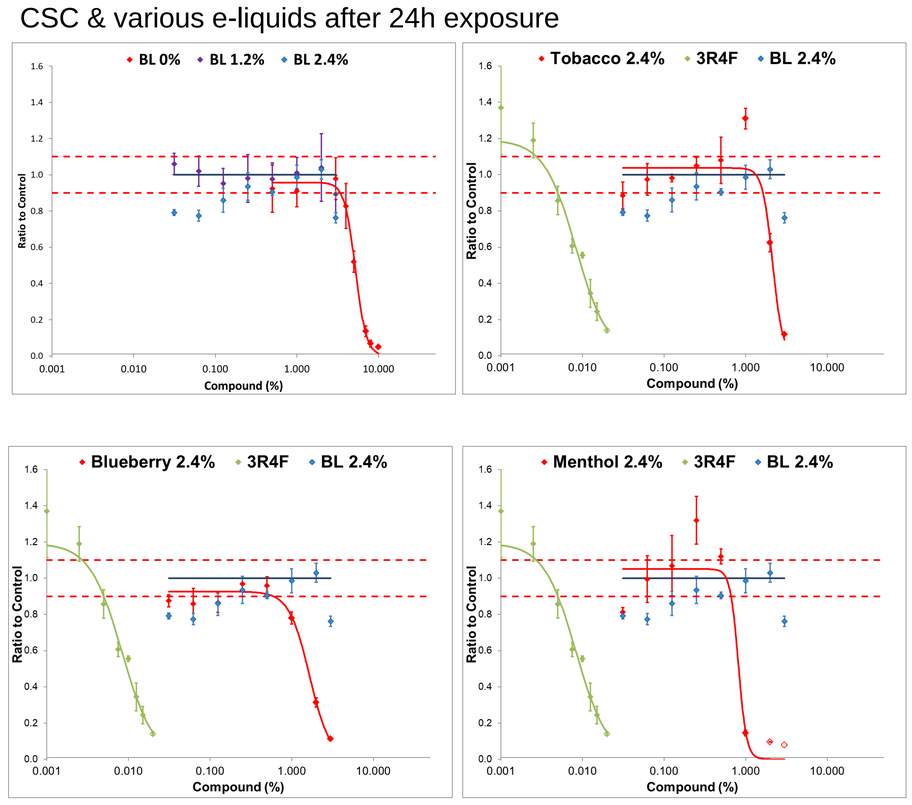


**Table 2:** Example test material osmolality per test concentration (mOsM/kg)

| Test Material Concentration (%) | 0.03125 | 0.0625 | 0.125 | 0.25 | 0.5 | 1 | 2 | 3 |
|---|---|---|---|---|---|---|---|---|
| BL 0% Nicotine Osmolality (mOsM/kg) | 0.295 | 0.299 | 0.308 | 0.325 | 0.364 | 0.438 | 0.599 | 0.753 |
| Test Material Concentration (%) | 0.001 | 0.0025 | 0.005 | 0.0075 | 0.01 | 0.0125 | 0.015 | 0.02 |
| 3R4F CSC Osmolality (mOsM/kg) | 0.2865 | 0.287 | 0.2875 | 0.2875 | 0.289 | 0.2895 | 0.2885 | 0.289 |

**Table 3**: Differences in MEC values between 3R4F CSC and Menthol e-liquid

| HCS parameter | Difference in MEC concentration (CSC vs Menthol 2.4% e-liquid) |
|---|---|
| Cell Count | 360 |
| Nuclear size | N/A |
| DNA Structure | N/A |
| Cell Cycle Arrest | 43 |
| Cell Membrane Permeability | 659 |
| Caspase 3/7 | 106 |
| NF-kB | 136 |
| Mitochondrial mass | 169 |
| Mitochondrial mem Pot | 130 |
| Oxidative stress | 170 |
| Glutathione content | 91 |
| Cellular ATP | 132 |

N/A: Not Applicable

The 3R4F CSC was the most biologically active test article, inducing most of the cellular endpoints at concentrations typically one hundred times lower (See Tables 1 & 3) than that for all the e-liquids. The BL e-liquids were largely inactive in most of the endpoints up to 3% test concentrations, with a tendency to lower MEC values with an increasing nicotine concentration (Figure 1). The commercial samples induced additional effects above those observed with the experimental e-liquids. For instance, the Menthol e-liquid was deemed to be the most active flavour. However, activity only occurred at concentrations significantly higher than the 3R4F CSC MEC (see Table 3). Menthol is known to cause cell cycle arrest in certain cell types (at 100 µM) [2]. Menthol has a proven record of safety, with a long history of use in pharmaceuticals (e.g. Nicotine Replacement Therapy) and cosmetic products[3]. Osmolality increased with increasing concentration of BLs (see Table 2) with no changes observed for increasing concentrations of 3R4F CSC, e-liquid flavors or nicotine (data not shown).

## 4. CONCLUSIONS

- Cigarette smoke condensate was the most biologically active test article, inducing effects from a concentration of 0.001%.
- For the e-liquid samples, effects were seen at concentrations typically 100 times higher than those for 3R4F CSC.
- Out of the e-liquid samples, Menthol was the most active flavour, but only at concentrations significantly higher than 3R4F CSC. Menthol has long history of use in a pharmaceutical products and a proven safety record.
- The high exposure concentrations of neat e-liquids, plus the exposure duration of 24 hours are an exaggeration of any potential human exposure.
- Work is underway to determine local cellular concentrations of e-liquid aerosol in 3D cells, when exposed at the air-liquid interface.

## REFERENCES

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2) Wang *et al*., (2012) Path Oncol Res, 18, pages 903-910.
3) Heck (2010), Food Chem Toxicol.; 48, Suppl 2:S1-38