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High Content Screening in NHBE cells shows significantly reduced biological activity of flavoured e-liquids, when compared to cigarette smoke condensate

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ABSTRACT

There is scientific agreement that the detrimental effects of cigarettes are produced by the formation of Harmful and Potentially Harmful Constituents from tobacco combustion and not by 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.

In this study, high content screening (HCS) was used to compare the effects of neat e-liquids and 3R4F reference cigarette smoke condensate (CSC), which served as a positive control, in Normal Human Bronchial Epithelial (NHBE) cells. The endpoints measured covered cellular health, energy production and oxidative stress. Base liquids, with or without nicotine, and commercial, flavoured, nicotine-containing e-liquids (CFs), had little or no effect on cell viability and most HCS endpoints even at significantly higher concentrations (typically 100 times or higher) than 3R4F CSC. CSC induced a dose-dependent decrease of cell viability and triggered the response in all HCS endpoints. Effects of CFs were typically observed at or above 1%. CF Menthol was the most active flavour, with minimum effective concentrations 43 to 659 times higher than corresponding 3R4F CSC concentrations. Our results show a lower biological activity of e-liquids compared to cigarette smoke condensate in this experimental setting, across wide range of cellular endpoints.

1. Introduction

The use of electronic cigarettes (e-cigarettes) is increasing worldwide by smokers seeking less harmful alternatives. Many in the public health community have concluded that e-cigarettes constitute a less harmful source of nicotine than cigarette smoking (Farsalinos et al., 2013a; Goniewicz et al., 2014; Misra et al., 2014; McNeill et al., 2015; Bullen et al., 2013; Brown et al., 2014). Most e-liquids are typically composed of a propylene glycol (PG) and/or vegetable glycerin (VG) base, in different ratios, with or without nicotine, and varying flavouring substances (Brown, 2014). Flavours play a critical role in attracting, and retaining smokers to e-cigarettes (Farsalinos et al., 2013b; Russell et al., 2018). As such, the market for flavoured e-liquids is rapidly expanding (Bullen et al., 2013; Zhu et al., 2014). The sheer diversity of e-liquids on the market has made it difficult to comprehensively study e-liquids, and to date, little systematic research has been conducted to assess their safety. Some studies have suggested that e-liquids may have measurable biological effects on cells, including effects on cell growth, viability, and inflammation, and the role of flavourings in these effects is being questioned (Rowell et al., 2016; Behar et al., 2014; Sherwood and Boitano, 2016; Sassano et al., 2018).

There is therefore a requirement for the development of standardised toxicity assessment methods to fill data gaps, to add to a weight-of-evidence approach for the risk assessment of e-liquids and their ingredients, support product development, stewardship and as a viable alternative with potentially reduced risk for adult smokers (Hartung, 2016).

The conducting airway epithelium provides the first line of defense against inhaled particulates, pathogens, allergens, and other noxious agents. A compromised airway epithelium can lead to infection, inflammation and airway remodeling associated with the onset and pathogenesis of chronic lung disease (Bals and Hiemstra, 2004). As

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cigarette smoke and e-cigarette aerosols are delivered directly to the airway, airway epithelial cellular models are considered to be suitable to assess their relative potential toxicity. In this study, we used Normal Human Bronchial Epithelial (NHBE) cells that have been previously used to assess the toxicity of cigarette smoke and novel tobacco and nicotine products (Fields et al., 2005; Kogel et al., 2015; Gonzalez-Suarez et al., 2017). We assessed neat e-liquids and cigarette smoke condensate (CSC) to determine biological activity in this cell system.

High content screening (HCS) is a method used in biological research and drug discovery to rapidly and cost-effectively screen multiple compounds for biological activity and toxicity and to elucidate possible mechanistic pathways in intact biological systems. HCS monitors and quantifies multiple cellular and subcellular endpoints simultaneously in a single assay through fluorescent dyes and tags and automated image analysis (Dorval et al., 2018). Phenotypic observations may include changes in cellular and subcellular morphology, protein expression, localization, and post-translational modifications. It has been proposed that HCS using primary human cell cultures could form a key part of a framework for the toxicological assessment of e-liquids and their aerosols that avoids the use of animals (Iskandar et al., 2016). HCS has previously been used to assess the toxicity of cigarette smoke constituents (Marescotti et al., 2016), heated tobacco products (Kogel et al., 2015; Taylor et al., 2017) and e-liquids (Sassano et al., 2018; Gonzalez-Suarez et al., 2017).

This paper is part of a wider framework of pre-clinical *in vitro* toxicity testing (see Fig. 1) and is aligned with the principles of the 3R's and the 21st Century Toxicology framework, in which animal use should be minimized or no longer required (Berg et al., 2011; Rovida et al., 2015; Sheldon and Cohen Hubal, 2009). With the focus of increased relevance of human primary cells-based *in vitro* assays. In this study, we compared the biologicals effects of experimental and commercial e-liquids and cigarette smoke condensate (CSC) generated from the 3R4F reference cigarette, with a particular focus on the potential impact of nicotine and flavours on the endpoints associated with general cellular health, energy production and oxidative stress. For this purpose, multi-parametric indicators of cellular toxicity were measured over a range of concentrations after 24 h of continuous exposure.

2. Material and methods

2.1. Control and test agents

The test agents were 3R4F CSC and seven e-liquids: three experimental (manufacturer Fontem Ventures, Amsterdam) and four commercially available flavours purchased from the US market (blu®). The compositions of the e-liquids are reported in Table 1. The composition of the CFs (commercial flavoured e-liquids) were analysed by GC–MS (n = 2).

Table 1
Composition of test agents.

Test agents[a]	Content [w/w %]			
	Propylene glycol (PG)	Vegetable glycerol VG	Nicotine	Flavourings and water
BL 0%[a]	50	50	–	–
BL 1.2%[a]	49.4	49.4	1.2	–
BL 2.4%	48.8	48.8	2.4	–
CF Tobacco 2.4%[a]	36.3	55.8	2.4	5.5
CF Menthol 2.4%[a]	29.3	63.2	2.4	5.1
CF Blueberry 2.4%[a]	55.8	39	2.4	2.8
CF Vanilla 2.4%[a]	39.6	56.6	2.4	1.7

BL: base liquid; CF: commercial flavoured e-liquid.
[a] % refer to % nicotine content.

All cigarette smoke collections were performed with an ISO harmonized Borgwaldt RM-20 D smoking machine. Cigarettes were conditioned according to ISO standard 3402 (International Organization for Standardization, ISO 3402, 1999).Total smoke particulate matter (TPM) was collected on a 92-mm Cambridge filter pad (CFP). Health Canada Intense modified (HCIm) smoking regime (55 mL/2 s/30 s without blocking of filter ventilation) was used directly after smoking, the filter pad was cut radially with a scalpel in 6 identical parts by using a special cutting device guide. Two opposite sections were used for nicotine and water evaluation of the CSC. The CSC from the remaining 4 parts of the filter pad were extracted in 13.3 mL dimethylsulfoxide (DMSO) by shaking for 20 min at room temperature. The extracts were centrifuged through sterile PP-filter (0.45 µm) in Maxi-Spin Filter Tubes (CIRO) and frozen at −70 °C aliquoted in 0.75 mL portions. The CSC concentration in DMSO was adjusted to 50 mg of TPM/mL, which corresponded to 1.76 cigarettes per mL of DMSO.

Test concentrations were determined based on sample activities and toxicities determined in previous research (data not shown). 3R4F CSC was tested at 0.001, 0.0025, 0.005, 0.0075, 0.01, 0.0125, 0.015, and 0.02%; BL 0% was tested at 0.5, 1, 3%; and CF e-liquids were tested at 0.0313, 0.0625, 0.125, 0.25, 0.5, 1, 2, and 3%. All test agents were diluted in PBS to make a stock solution, which was further diluted with

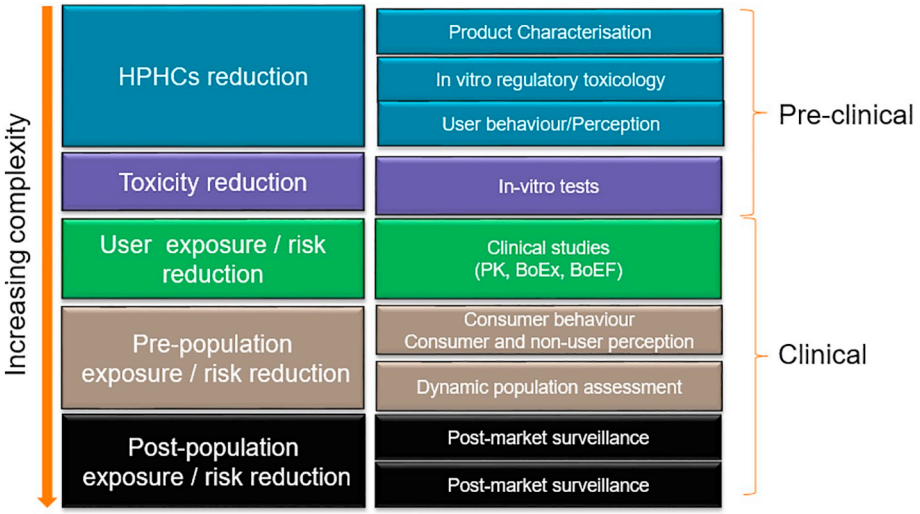

Fig. 1. Overview of Imperial Brands Harm Reduction Approach, indicating where the *in vitro* tests are used within the overall framework. Abbreviations: Pharmacokinetics PK, Biomarkers of exposure BoEx; Biomarkers of Effect BoEF

PBS. Each of the final concentrations were added to the assay media at a concentration of 10%.

2.2. Dosimetry

Nicotine is routinely used as marker for quantification of exposure to cigarettes and next generation products (Adamson et al., 2017; Behrsing et al., 2018). The dose of nicotine at the highest concentration of e-liquid tested (3%) corresponds to a cellular exposure of 720 mg mL for the 2.4% nicotine content. However, human nicotine measured plasma concentrations for cigarette smoking are reported to range from 10 to 37 ng/mL (Schneider et al., 2001).

2.3. Measurement of osmotic concentrations

Both PG and VG can increase the osmotic concentration of a solution and can induce hyperosmotic shock in exposed cells, eventually leading to cytotoxicity (Gonzalez-Suarez et al., 2017). To identify potential cytotoxic effects due to hyperosmolality, the osmotic concentration of all test solutions used to expose cell systems were measured (see Table 3).

The total osmotic concentration was determined with use of Osmomat 030 (Gonotec). Each sample (50 µL) was added to a vial and inserted into the machine for comparative measurements of the freezing points of pure water and test solutions. Whereas water has a freezing point of 0 °C, a solution with saline concentration of 1 Osm/kg has a freezing point of −1.858 °C. All measurements were made in triplicate.

2.4. Cell culture

Normal Human Bronchial Epithelial (NHBE) cells (Lonza, Cologne, Germany) were cultured in Airway Epithelium Cell Growth Medium (PromoCell). The cells were bronchial epithelial cells extracted from healthy human donors, being human relevant primary cells. The use of the cell growth media kept the cells at the same state, to enable relative comparison across treatments and across time. The metabolic capacity of the cells was not measured but was considered to be low, when compared to fully differentiated 3D models (Boei et al., 2017). The cells were subcultured every 6 to 9 days until passage 9. Briefly, cells were detached using the subculture ReagentPack™ (Lonza), subsequently cells were centrifuged at 1200 rpm for 5 min, re-suspended in media and counted. The cells were seeded at standard culture conditions in T175 cell culture flasks and allowed to grow until 85% confluence at 37.0 °C in a humidified incubator with a 5% CO_2 atmosphere. After the growth period, the cells were seeded for use in the designated assays.

2.5. NHBE cell health markers analysis using high-content imaging

Black walled, clear bottomed polystyrene 96-well cell culture plates (Corning) were seeded with 9000 NHBE cells per well followed by a 24-h incubation at 37 °C and 5% CO_2. NHBE cells were then exposed to a serial dilution, using an eight-point concentration range, three replicates per concentration and endpoint of the controls and test articles, for a duration of 24 h before running the HCS assays. The exposure duration was selected based on previous research showing no effect of e-liquids at concentrations up to 0.5% after 4 and 24-h exposures, and more pronounced effects at higher concentrations observed at the 24-h timepoint (data not shown). At the end of the incubation period, cells were stained with the specific dye/antibody for each HCS endpoint.

High content imaging was performed using an automated fluorescent cellular imager with either a Cellomics® ArrayScan VTI or a Cellomics® ArrayScan XTI HCS instrument (Thermo Fisher UK), which utilised HCS Studio 2 software (Thermo Fisher) for image analysis. A minimum of eight individual images were acquired per fluorescent channel for each well of the experimental plates.

The cell health markers used were cell count, cell cycle arrest (Hoechst 33342; Sigma or Syto11; Molecular Probes), mitochondrial mass and potential (MitoTracker® Deep Red; Molecular Probes), oxidative stress (Dihydroethidium; Sigma), glutathione content (monochlorobimane; Sigma), cell membrane permeability (TO-PRO-3, Thermofisher Scientific), Caspase 3/7 activity (CellEvent Caspase-3/7 green, Thermofisher Scientific) and NF-κB (Rabbit Ab, Invitrogen). Cellular ATP was determined using luminescence following the manufacturer's guidelines (CellTiterGlo, Promega) and luminescence was determined using a BioTek Synergy 2 (BioTek).

Raw fluorescence intensity values (RFU) were normalised to vehicle control in all cases and expressed as fold changes in assay signal. Cellular responses to BLs, CFs or 3R4F CSC of an individual HCS endpoint was considered biologically relevant, only if the fold change response was greater than 1.2 or less than 0.8 relative to vehicle control.

2.6. Data display for HCS endpoints

For all of the HCS endpoints we have focused on the MEC (Minimum Effective Concentration), which is the lowest concentration that significantly crosses the vehicle control threshold, (see Fig. 2 A-C, for an example). We also reported the $AC_{50,}$ concentration, which is the concentration at which 50% maximum effect was observed for each of cell health parameter. The red and green colouring in Table 4 are to enable an easy interpretation of the results. Lower MEC values were graded towards red (effects outside the vehicle control observed with lower concentrations) and higher concentrations towards green (biological response observed at higher concentrations, or no response up to the highest concentration tested). The white colour indicates values for specific endpoints in which MEC is close to 1% (based on cell concentrations that have no cytotoxicity associated in this and in other human primary cell based assays we have used, (data not shown)). The individual HCS endpoints assessed in this study are presented in Table 2.

3. Results

3.1. Osmotic concentrations

The osmotic concentrations of the control and test solutions are reported in Table 3. As expected, the osmotic concentrations increased with increasing concentrations of test agents. With a typical physiological level for airway cell cultures at 315 ± mOsm/kg, values in the range of 300–400 mOsm/kg were considered to be within physiological range for this cell culture. The osmotic concentrations of 3R4F CSC and e-liquid solutions at, and above, 2% exceeded typical physiological osmolality levels. However, there was no effect of BL 0% (except a small decrease of cellular ATP) at concentrations up to 3% corresponding to an osmolality of 753 mOsm/kg in the HCS assay. When the same e-liquids were tested in another human primary cell system (BioMap®) consisting of 12 panels of human primary cells in co-culture, the maximal concentration tested was 1% due to cytotoxicity being seen at higher concentrations (publication in preparation).

3.2. Cell count

Decreased cell count is an indicator either of cell death (apoptosis, necrosis) or a reduction in cellular proliferation. A dose-dependent decrease in cell count was observed upon 3R4F CSC exposure. At the highest concentration tested (0.02%), cell count was decreased by approximately 80% for CSC. In the absence of nicotine, BL 0% had no effect on cell count at any of the tested concentrations up to 3%. The addition of nicotine did not increase the cytotoxicity of the base liquid, except for BL 2.4% when tested at 3%. Three CFs (Tobacco, Vanilla and Blueberry) were not cytotoxic up to 1%, with decreased cell counts

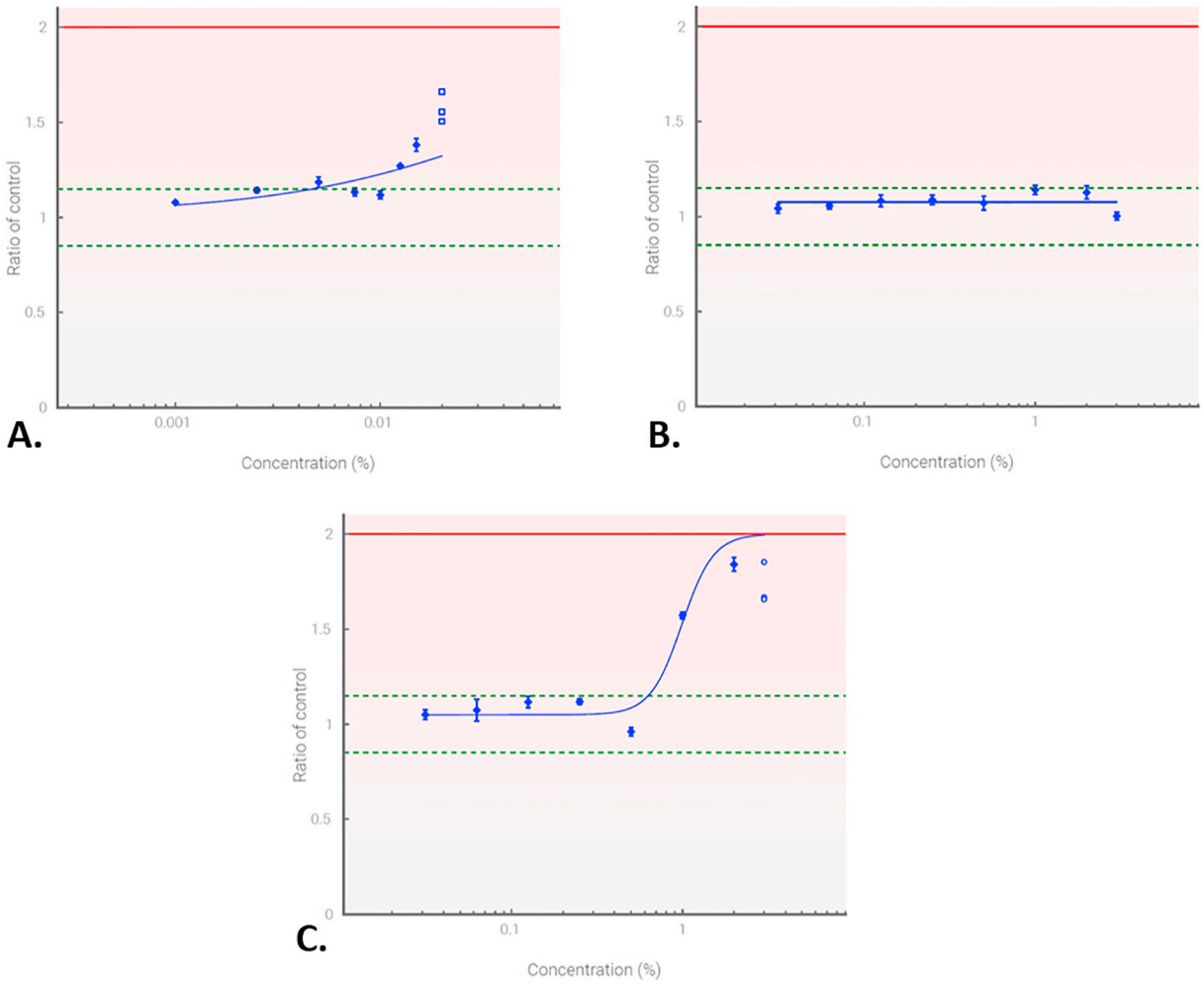

Fig. 2. Representative plot of NF-κB endpoint expressed as a substance testing concentration vs ratio of control indicating at which concentration the test substance departed the vehicle control envelope. A. 3R4F CSC (0.001–0.02%); B. BL 2.4% (0.0313–3%); C. CF Menthol 2.4% (0.0313–3%). Green dashed lines represent a significant cut-off from vehicle control (used to calculate the MEC). Red solid line indicates maximum and minimum responses, used to calculate AC_{50}. Filled blue dots represent data points for each concentration (plus or minus standard deviation). Open blue circles represent data points excluded from plot due to data plateau, or other reasons. Open blue squares represent data points excluded from plot due to cell loss or nuclear size. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

observed only at higher concentrations. CF Menthol had a lower MEC (Minimum Effective Concentration: the concentration that crosses the vehicle control threshold values in this cellular endpoint) for cell count than the rest of CFs. All of these effects occurred at osmotic concentrations above the physiological range (see Tables 2 and 4). Also, any observed cytotoxicity of CFs occurred at much higher concentrations than 3R4F CSC (see Table 4) and after 24h of continuous exposure.

Table 2
Location of cellular compartment used for the quantification within specific HCS endpoints.

Assay	Endpoint	Biological endpoint	Cellular Compartment	Output feature used for quantification
Necrosis, Apoptosis, NF-κB and Cell Cycle Arrest Assay	1	Cell count	Nucleus	Object count
	2	Cell cycle arrest	Nucleus	Average intensity ratio
	3	Cell membrane permeability	Nucleus	Average intensity
	4	Caspase 3/7 intensity	Cytoplasm	Spot average intensity
	5	NF-κB	Nucleus	Average intensity
GSH, ROS, MMP & ATP Assay	6	Cell count	Nucleus	Object count
	7	Mitochondrial mass	Cytoplasm	Spot average intensity
	8	Mitochondrial membrane potential	Cytoplasm	Spot total area
	9	Oxidative stress	Nucleus	Average area
	10	Glutathione content	Cytoplasm	Average intensity
	11	Cellular ATP	Whole cell	Well

Table 3
Osmotic concentrations of control and test solutions (Osm/kg).

Concentration (%)	0.03125	0.0625	0.125	0.25	0.5	1	2	3
Test agent								
BL 0%	0.295	0.299	0.308	0.325	0.364	0.438	0.599	0.753
BL 1.2%	0.295	0.300	0.308	0.327	0.368	0.449	0.621	0.738
BL 2.4%	0.296	0.302	0.318	0.332	0.375	0.459	0.631	0.774
CF Vanilla 2.4%	0.297	0.298	0.306	0.327	0.364	0.438	0.594	0.701
CF Menthol 2.4%	0.298	0.299	0.311	0.334	0.371	0.439	0.586	0.701
CF Tobacco 2.4%	0.296	0.303	0.313	0.334	0.372	0.443	0.588	0.702
CF Blueberry 2.4%	0.298	0.298	0.308	0.331	0.375	0.451	0.618	0.747
Concentration (%)	0.001	0.0025	0.005	0.0075	0.01	0.0125	0.015	0.02
3R4F CSC	0.2865	0.2875	0.2875	0.2875	0.289	0.2895	0.2885	0.289
DMSO	0.287	0.2875	0.288	0.2885	0.2885	0.288	0.289	0.2885

3.3. Cell cycle arrest

DNA damage and other cell damages can trigger cell cycle arrest at cell cycle checkpoints through the regulation of the activities of a family of protein kinases known as the cyclin-dependent kinases (Barnum and O'Connell, 2014). Cell cycle arrest was determined as the ratio of G0/G1 to G2/M. An increase in this endpoint signal was therefore linked to G0/G1 arrest and a decrease to G2/M arrest (DNA damage). All test samples triggered G0/G1 cell cycle arrest. Exposure to 3R4F CSC triggered a dose-dependent increase in G0/G1 cell cycle arrest. Exposure to nicotine-containing BLs also triggered a dose-dependent increase in G0/G1 cell cycle arrest, although at much higher concentrations than 3R4F CSC, i.e., at and above 1%. Exposure of the NHBE cells to CFs resulted in an increase of cell cycle arrest at concentrations lower than observed for BLs containing nicotine (see Table 4). CF Menthol was active at lower concentrations (MEC derived as 0.0551%) than the other CFs, but at higher concentrations than 3R4F CSC (see Table 5).

3.4. Cell membrane permeability

Increased cell membrane permeability is an indicator of late apoptosis or necrosis. A dose-dependent increase in cell membrane permeability was observed upon 3R4F CSC exposure (even the lowest dose tested was above the control envelope). Nicotine-free BL had no effect on cell membrane permeability relative to controls at the concentrations tested. In the presence of nicotine, small but significant increases in cell membrane permeability was observed already at the lower concentrations, however, they were much smaller in magnitude, and occurred at much higher concentrations, than the one of 3R4F CSC. Exposure to CFs triggered dose-dependent increases in cell membrane permeability. Most of the effects were observed above 1%, with CF Menthol and CF Blueberry increasing the cellular membrane permeability at slightly lower concentrations (MEC of 0.659% and 0.783% respectively) than CF Vanilla and Tobacco (MEC of 1.64 and 1.52% respectively). All of these effects occurred at much higher concentrations than the ones of 3R4F CSC (see Table 4).

3.5. Caspase 3/7 activity

Sequential activation of members of the cysteine aspartic acid-specific protease (caspase) family plays a central role in the execution of cell apoptosis (Thornberry et al., 1997). An increase in caspase 3/7 activity indicates the onset of the cell signaling cascade leading to apoptosis. Up to 3%, BLs, with and without nicotine, had no effect on caspase 3/7 activity relative to control. A dose-dependent increase in caspase 3/7 activity was observed upon exposure to 3R4F CSC and to CFs, although at much higher concentrations for CFs. CF Blueberry and Tobacco had the MEC of 0.596 and 0.652% respectively. CF Vanilla had no effects on the caspase 3/7 activity up to 1% testing concentration. CF Menthol, having a minimum effective concentration 100 times higher than 3R4F CSC, was the most active of the CFs (see Table 5).

3.6. Nuclear factor-κB (NF-κB)

The transcription factor NF-κB regulates multiple aspects of innate and adaptive immune functions and serves as a pivotal mediator of inflammatory responses. In an inactivated state, it is located in the cytosol, complexed with the inhibitory protein I-κB. Upon cellular stress the complex dissociates and NF-κB translocates into the nucleus, where it triggers the expression of various proinflammatory genes, including cytokines, chemokines, and adhesion molecules (Liu et al., 2017). An increase in NF-κB is therefore an indicator of cellular stress and inflammatory process (see Fig. 2). Up to 3%, BLs, with and without nicotine, had no effect on NF-κB relative to the controls (see Table 4). A dose-dependent increase in NF-κB was observed upon 3R4F CSC exposure (see Fig. 2). Exposure to CFs also increased NF-κB, but only at concentrations higher than 1%, except for CF Menthol increasing the NF-κB translocations from a concentration of 0.63%. This was still in excess of 130 times higher than the effective concentration for 3R4F CSC.

3.7. Mitochondrial mass

Increases in mitochondrial mass and mitochondrial DNA content are early events of the cellular response to endogenous or exogenous oxidative stress (Lee et al., 2000). An increased mitochondrial mass can also indicate mitochondrial swelling further to mitochondrial membrane potential collapse (Hosseini et al., 2013). A decrease in mitochondrial mass indicates loss of total mitochondria further to loss of mitochondrial integrity. A dose-dependent increase in mitochondrial mass was observed upon exposure to 3R4F CSC with the MEC of 0.006% (see Table 4). Up to 1%, BLs (without and with 1.2% nicotine) had no effect on mitochondrial mass relative to control. The decrease of mitochondrial mass at 0.8% observed for BL 2.4% was deemed not significant. CFs Blueberry, Tobacco and Vanilla had little to no effect on the mitochondrial mass. Above 0.88% CF Menthol increased the mitochondrial mass relative to control. The 3R4F CSC had an MEC value 169 times lower than CF Menthol.

3.8. Mitochondrial membrane potential

Apoptotic stimuli can trigger changes in the inner mitochondrial membrane that result in an opening of the mitochondrial permeability transition pores resulting in decreased mitochondrial membrane potential and release of a number of cell death-promoting factors from the mitochondrial inter-membrane space to the cytosol. For example, one group of proteins that is released include cytochrome *c*, Smac/DIABLO,

Table 4
Summary of the minimum effective concentrations (MEC) and AC_{50} values (%) for all the endpoints, obtained for the 3R4F CSC and e-liquid.

	High Content Screening - Biological Response Heat Map															
	3R4F (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
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	0.02ǂ	NR	NR	↑0.345 (NS)	>3ǂ (NS)	↑0.149	>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: the concentration that crosses the vehicle control threshold values. NR = no response observed. 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 an increase or decrease for specific cellular endpoint. BL = Base liquid 50:50 PG/VG with nicotine %. NS = This indicates that the value was not significant in terms of curve fitting. The red and green colouring are to enable and easy interpretation of the results. White colour = values for specific endpoints in which MEC is close to 1% (based on cell concentrations that have no cytotoxicity associated in this and in other human primary cell based assays we have used). Lower MEC values are graded towards red (effects outside the vehicle control observed with lower concentrations) and higher concentrations towards green (biological response observed at higher concentrations, or no response up to the highest concentration tested). 3R4F CSC was tested between 0.001 and 0.02% and the e-liquids between 0.0313 and 3% based on previous work (data not shown).

Table 5
The differences in the Minimum Effective Concentration (the concentration outside the control envelope) between 3R4F CSC and CF Menthol 2.4% (the most biologically active CF).

HCS parameter	Difference in MEC concentrations (CF Menthol 2.4% divided by 3R4F CSC)
Cell count	360
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

and the serine protease HtrA2/Omi which can initiate the caspase-dependent cell death pathway (Elmore, 2007). No change in mitochondrial membrane potential relative to control was detected upon exposure to BLs, with or without nicotine, up to 3%. A dose-dependent decrease in mitochondrial membrane potential was observed upon 3R4F CSC exposure even at very low testing concentrations (MEC of 0.005%). Exposure to CF Blueberry, Tobacco and Menthol also decreased mitochondrial membrane potential in a dose-dependent manner, with concentrations outside of the vehicle control (MEC) only at above 0.6% testing concentration. At concentration above 1% CF Vanilla had a similar effect. These concentrations were at least 120 times higher than the corresponding 3R4F CSC concentrations (see Tables 4 & 5).

3.9. Reactive oxygen species (ROS) formation

ROS are formed as natural by-products of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. The main source of ROS is mitochondrial respiration. ROS levels can increase dramatically further to stress and with continued exposure and/or high levels of ROS, apoptosis mechanisms are triggered (Simon et al., 2000). No change in ROS formation relative to control was observed upon exposure to BLs, with or without nicotine, up to 3% (see Table 4). A dose-dependent decrease in ROS formation was found after exposure to 3R4F CSC, probably due to cell death. Shorter timepoint (4 h) will be used in future studies to possibly capture early cellular events. Exposure to the remaining CFs also triggered a dose-dependent decrease in ROS formation, although the effects occurred at much higher concentrations (0.8% and above), with MEC approximately 170 times higher than 3R4F CSC.

3.10. Reduced glutathione (GSH) content

GSH is the key antioxidant in animal tissues. GSH can prevent damage to important cellular components caused by ROS. Glutathione reduces disulfide bonds formed within cytoplasmic proteins to cysteines by serving as an electron donor. In the process, glutathione is converted to its oxidized form, glutathione disulfide (GSSG). Once oxidized, glutathione can be reduced back by glutathione reductase, using NADPH as an electron donor. An increase in GSH represents an adaptive cellular response to oxidative stress. In turn, increased oxidative stress may lead to intracellular depletion of GSH and a decrease in GSH content suggests the presence of oxidative stress (Park et al., 1998; Rahman and MacNee, 1999). Exposure to cigarette smoke has been shown to result in GSH depletion in the lung cells both *in vitro* and *in vivo* (Park et al., 1998; Rahman and MacNee, 1999; Baglole et al., 2006). Of note, cigarette smoke-induced depletion of intracellular GSH is thought to occur without concurrent increase in GSSG (Rahman et al., 1995) possibly due to an irreversible formation of GSH conjugates that cannot be reduced (van der Toorn et al., 2007a).

No change in GSH content relative to control was detected upon exposure to BLs, with or without nicotine, up to 3%. A dose-dependent decrease in free GSH was found after exposure to 3R4F CSC. A decrease of the glutathione content was observed after exposure to CF Tobacco, Blueberry and Vanilla, although at much higher concentrations (only above 1%). CFs Menthol caused a decrease of the GSH content at concentration lower than for the rest of CFs (see Table 4). The concentration of CF Menthol was, however, 90 times higher than the concentration of 3R4F CSC that caused a decrease in GSH content of the cells.

3.11. Cellular ATP

Mitochondria are the major site of oxidative metabolism, and hence ATP synthesis, which is tightly controlled by intramitochondrial Ca^{2+} in a mechanism designed to ensure that ATP synthesis is closely coupled to the cell's energetic needs (Tarasov et al., 2012). A decrease in cellular metabolic activity therefore results in a decrease in the level of ATP detected. However, ATP depletion can also result from mitochondrial dysfunction further to stress-related mitochondrial membrane potential loss. Cigarette smoke exposure is known to inhibit the energy generation process in mitochondria, with reduced oxidative phosphorylation and ATP generation (van der Toorn et al., 2007b; Kennedy and Elliott, 1970; Gairola and Aleem, 1973). A dose-dependent decrease in cellular ATP was found after exposure to 3R4F CSC. A dose-dependent decrease in cellular ATP also occurred after exposure to BL 0%, although effects occurred at much higher concentrations, above 2.5%. Addition of nicotine tended to lower the cellular ATP, but only at concentrations above 1% (see Table 4). CFs displayed similar dose-response curves as BL 2.4%, with values crossing the lower vehicle control line at slightly lower concentrations for CFs Menthol and Blueberry (MEC of 0.6 and 0.9%).

4. Discussion

In this study, we assessed the biological impact of the principle components of e-liquids (PG, VG and nicotine) as well as the effects of commercial flavourings on NHBE cells using the HCS methodology. Our study was designed to evaluate the biological effects of e-liquids and to compare them with the positive control reference cigarette 3R4F CSC in a range of cellular endpoints covering cellular health, energy production and oxidative stress. Lung epithelial cells were selected because they constitute the first biological barrier against inhaled toxicants.

We chose commercially available normal human bronchial epithelial cells and acknowledge that the endogenous metabolic capacity is probably very low (Boei et al., 2017). Several authors have measured the metabolic capacity of NHBE cells. At a genomic level NHBE cells were found to respond to tobacco smoke condensates, and Phosphate-buffered saline (PBS) trapped samples with significant upregulation of genes related to xenobiotic metabolism and the upregulation of antioxidants (Kogel et al., 2015; Jorgensen et al., 2004; Pickett et al., 2010). However, the genetic changes do not always correspond to protein changes at the cellular level. We agree with other authors, that these NHBE cells are clearly human-relevant and useful for screening purposes.

We focused on the use of the Minimum Effective Concentration (MEC), the lowest concentration (%) at which the various HCS parameters leave the control envelope. The AC_{50} corresponds to half of the maximum response recorded for the parameter as a percentage concentration and is also displayed in Table 4. Our results demonstrate a significantly lower biological activity and toxicity of e-liquids (typically in excess of 100 times less active) compared to CSC in NHBE cells in this experimental setting. Altogether, HCS in NHBE cells is a sensitive and physiologically relevant method to evaluate the toxicological effects of e-liquids.

The relevance of the e-liquid doses to NHBE cells was compared to human plasma levels of nicotine. Nicotine is routinely used as marker for quantification of exposure to cigarettes and next generation products (Adamson et al., 2017; Behrsing et al., 2018). In our study, the dose of nicotine at the highest concentration of e-liquid tested (3% in assay condition) corresponds to the dose of 720 mg/mL of culture media (estimated for 2.4% nicotine containing e-liquids). Human nicotine plasma concentrations for cigarette smoking are reported to range from 10 to 37 ng/mL (Schneider et al., 2001; Hukkanen et al., 2005). In a recent study 15 volunteers vaped e-cigarette (myblu™) with e-liquid of matching nicotine strength (25 mg/mL) to the one used in our study, for 10 puffs, of 3 s duration every 30 s, this gave rise to a mean plasma nicotine concentration of 5 ng/mL (O'Connell et al., 2019). The nicotine levels NHBE cells were exposed to for our HCS analysis, are is excess of typical human doses achievable under either normal or excessive usage and represented exaggerated exposure.

4.1. 3R4F cigarette smoke condensate

3R4F CSC had a dose-dependent impact on cell viability and all HCS endpoints. 3R4F CSC exposure triggered typical oxidative stress responses, such as a decrease in mitochondrial membrane permeability, decreased ATP production, increased caspase 3/7 activity and GSH depletion. 3R4F CSC exposure also triggered inflammatory processes, as indicated by an increase NF-κB translocation to the nucleus. These findings are in line with previous findings of Kogel et al., who also assessed the biological impacts of the 3R4F reference cigarette on NHBE cells using HCS (Kogel et al., 2015). The authors found that exposure to 3R4F resulted, in amongst others, decreased cell count linked with both apoptosis and decreased cell proliferation, decreased cell membrane permeability, increased caspase 3/7 activation, and decreased free GSH amount. In both Kogel et al. and our study, the effects of cigarette smoke were dose-dependent, suggesting that toxicity mechanisms other than hyperosmotic shock were contributing.

The results for three endpoints were directionally different between Kogel et al. and our study. First, Kogel et al. found that 24 h of exposure to 3R4F increased the amount of ROS in NHBE cells, while we observed a CSC-induced decrease in ROS. Cigarette smoke is a known inducer of oxidative damage in cells due to increased ROS formation and increased ROS levels would be expected (Baglole et al., 2006; Rao et al., 2016). However, the change in ROS levels was shown to be time-dependent, with significant increases followed by a decrease (Rao et al., 2016), which could possibly be explained either by ROS trapping by GSH and/or by a lower ROS production due to cell death. Second, in Kogel et al., the mitochondrial membrane potential did, unexpectedly, not decrease. The authors hypothesize that this may be due to a targeted degradation of the mitochondria with lower membrane potentials, so that only mitochondria with high membrane potential remained. Finally, we observed a dose-dependent increase, in mitochondrial mass upon exposure to 3R4F CSC. One explanation for our observed increase may be mitochondrial swelling further to membrane potential loss, or osmotic effects at the intra-cellular level.

Experimental and commercial e-liquids were tested at much higher concentrations than 3R4F CSC. Yet, many of the observed effects upon exposure to 3R4F CSC were not observed with e-liquids, and some were only observed at the highest e-liquid concentrations. In the present study, a direct comparison of the concentrations used is difficult because we tested cigarette smoke condensate and e-liquids, not their aerosols, which might be more relevant to consumers. This study represents our first attempt to evaluate the sensitivity and the applicability of the HCS for the screening and stewardship purposes. Subsequent studies will involve the exposure to the e-cigarettes aerosols at the air-liquid interface to model the real user exposure. It has been found previously that, when comparing cigarette smoke and e-cigarette aerosols on the basis of the same smoking parameters and number of puffs, the cell viability was about 4.5–5 times lower and the oxidative stress levels 4.5–5 times higher in combustible cigarettes (Scheffler et al., 2015).

4.2. PG/VG mixture (BL 0%)

Both PG and VG are used extensively in pharmaceutical and cosmetic products, and are approved food ingredients used in food preparations. Their usage as individual ingredients have shown to have low systemic toxicity upon oral, dermal and inhalation exposures (Werley et al., 2011; Phillips et al., 2017; Walele et al., 2018; Joint, 2001).

We carried out a full-dose response, up to 3%, for the nicotine-free e-liquid BL 0% in order to evaluate the upper biological response to PG/VG with the purpose of creating a baseline for CFs. The only finding was a slight reduction in the ATP production at concentrations above 2.59%. In this study we have evaluated the biological exposure of the NHBE cells directly exposed to neat e-liquid formulations for 24 h. It is very unlikely that exposures to e-liquid aerosols during normal consumption will reach cellular levels equivalent to continuous exposure for 24 h and concentrations of 1% e-liquids and above. At concentrations below 3%, BL 0%, had no impact on cell viability nor any HCS endpoints under conditions of test. Both PG and VG can have an effect on the osmotic concentration of a solution. In this study, a dose-dependent increase in osmotic concentration was observed for all BLs and CFs. At concentrations at and above 1%, the osmotic concentration of these samples, above 400 mOsm/kg, was above normal physiological levels and had the potential to induce hyperosmotic shock. Gonzalez-Suarez et al. also found that exposure of NHBE cells to PG and VG mixtures resulted in dose-dependent cytotoxicity and inhibition of cell proliferation, which they attributed, in part, to hyperosmotic shock (Gonzalez-Suarez et al., 2017).

In our study, we tested only a PG/VG 50:50 mixture (BL 0%). Gonzales-Suarez et al. assessed the relative contribution of PG and VG by exposing NHBE cells to different PG/VG mixtures (ratios from 0/100 to 100/0). They found dose-dependent decreases of the cell index, a measure of electrical impedance correlated with cytotoxicity, with all mixtures. However, EC_{50} values decreased with increasing PG content and the authors concluded that PG had a higher cytotoxic effect on NHBE cells than VG (Gonzalez-Suarez et al., 2017). Most of these effects, though, were generally observed only at high concentrations (1% and above).

4.3. Nicotine

With the addition of nicotine to the BL, some effects were observed at lower concentrations than in the absence of nicotine and appeared to be dose-related (cell cycle arrest, cellular ATP). However, most effects were still observed at or above 1% testing concentration. Similarly, to us, Gonzalez-Suarez reported that all PG/VG mixtures caused a dose dependent increase in cells entering cell cycle arrest (G0/G1), with their gene expression analysis revealing a significant activation of several cell cycling genes and p53 by mixtures of PG/VG and nicotine. Our study showed an effect of nicotine addition to PG/VG base formulation on cell cycle arrest leading to a dose-dependent increase in G0/G1 cell cycle arrest (Lee et al., 2005). This was also expected by Gonzalez-Suarez et al. but was reported as not being measured (Gonzalez-Suarez et al., 2017). Other HCS effects reported were at concentrations of PG/VG in excess of those presented in this paper (e.g. 4 and 8% v/v) (Gonzalez-Suarez et al., 2017).

In the Gonzales-Suarez et al. study, the presence of nicotine augmented the toxicity of all PG/VG mixtures when compared with their nicotine-free counterparts (Gonzalez-Suarez et al., 2017). Considering that nicotine did not increase the osmotic concentration of PG/VG mixtures and that the concentration of nicotine at EC_{50} values of the PG/VG mixtures was similar to the EC_{50} value for nicotine alone, the authors concluded that nicotine was the main toxicity driver in

nicotine-containing PG/VG mixtures and that different pathways were involved in osmotic shock and nicotine toxicity. Based on these findings, and our own findings on the lack of nicotine impact on the osmotic concentration of the PG/VG mixtures (see Table 2), we also hypothesize that nicotine effects were related to different mechanisms other than osmotic shock. This is clearly based on the HCS results seen where there are the features of the addition of nicotine to the BL and then additional effects of flavours in CFs. Further research in this area is required.

When comparing our results for BL with and without nicotine to Gonzalez-Suarez (Gonzalez-Suarez et al., 2017) there was a high degree of concordance with the corresponding data they had generated and ours. The only difference to our results was cell membrane permeability. With 1.8% nicotine PG /VG (50:50) Gonzalez-Suarez, did not see any effects up to 8%, with 2.4% nicotine we saw effects at 0.5% concentration.

Several studies have investigated the cytotoxic, genotoxic and carcinogenic effects of nicotine (Haussmann and Fariss, 2016). However, the results are heavily dependent on the species and cellular models used, thus causing inconsistency. Furthermore, few in vitro studies are available on primary human cells. Nicotine has been found to be cytotoxic in other human cell based assays. Bodas et al. reported that exposure to nicotine caused an increase in ROS, leading to increased senescence and apoptosis in the bronchial epithelial cell line BEAS-2B (Bodas et al., 2016). Nicotine decreased the cell metabolic activity and viability in a dose- and time-dependent manner in immortalized and malignant keratinocytes (Lee et al., 2005). Gonzales-Suarez et al. found that 24-h exposure of NHBE cells to nicotine doses above 5 mM activated biological pathways ultimately leading to apoptosis/necrosis, including a decrease in GSH content, increases in caspase 3/7 activity, cytochrome *c* release, p-H2AX levels, cell membrane permeability and mitochondrial health parameters (Gonzalez-Suarez et al., 2017). Conversely, a study in NHBE cells found no effect of nicotine on cell viability and only limited effects on oxidative stress levels after the exposure to aerosol of an e-cigarette liquid containing 2.4% nicotine compared to nicotine-free liquid (Scheffler et al., 2015).

In our study the addition of 2.4% of nicotine to the base e-liquid formulation lowered the cell count significantly only at the concentration above 2%. Changes in other HCS endpoints, except for cell cycle arrest and cellular ATP were deemed as not significant.

4.4. Commercial e-liquids (CFs)

The CFs differed from the nicotine-containing BLs by their humectant content ratios and by the presence of flavourings. However, they had similar nicotine contents and osmotic concentrations as BL 2.4%. CFs had dose-dependent effects on cell viability and most HCS endpoints, however with MEC typically above 1%, except for CF Menthol (see Table 4). Their impact generally occurred at lower concentrations than the impact of nicotine-containing BLs, but at much higher concentrations than those seen with 3R4F CSC. The four CFs had different impacts on different endpoints, both in terms of effective concentration and magnitude of effects. CF Menthol repeatedly showed higher activity than CFs Vanilla, Blueberry and Tobacco and is discussed in more detail below.

A growing number of studies are being performed on e-liquid flavourings with cytotoxicity as an endpoint, with variable results depending on the e-liquids, the cell systems and reported cytotoxic effects. Sassano et al. developed a high-throughput screening (HTS) assay and screened 148 e-liquid flavours in human embryonic kidney 293 (HEK293T) cells (Sassano et al., 2018). They reported that e-liquids affected cell viability in a dose-dependent manner with LC_{50} ranging from 0.14 to 6.00 (%v/v). Rowell et al. tested the effects of 13 different flavoured e-liquids on a lung epithelial cell line (CALU3) and found that all flavours tested induced a dose-dependent decrease in cell proliferation/viability, with some flavours showing significantly greater toxicity compared with the PG/VG control, indicating the potential for some flavours to elicit more biological effects than others (Rowell et al., 2017). The cytotoxicity of 33 e-cigarette refill fluids and three do it yourself products were tested on human embryonic stem cells (hESC), mouse neural stem cells (mNSC), and human pulmonary fibroblasts (hPF) and varied significantly from non-cytotoxic to highly cytotoxic. The authors concluded that cytotoxicity was not due to nicotine, but was correlated with the number and concentration of flavouring chemicals (Bahl et al., 2012). Human foetal lung fibroblast (HFL1) viability was not significantly affected following treatments with humectants or 3 commercial flavoured e-liquids compared with control after 24 h, unless they were cultured in small wells with fewer number/density of cells (Lerner et al., 2015). A study examining the impact of common e-liquid flavouring chemicals on immortalized human bronchial epithelial cells (16HBE14o-) also found that individual flavouring chemicals varied in their cytotoxicity profiles (Sherwood and Boitano, 2016).

As in this manuscript, these studies, tested e-liquids. However, the main consumer exposure to the e-liquid during normal use is to its aerosol. The aerosolizing process involves a brief heating period during every puff and the compounds can respond in varying degrees to the different processes involved in aerosolisation, such as evaporation and condensation. Together, these factors might result in changes to the composition of the aerosol versus that of the neat, un-aerolised e-liquid. Given the numerous and evolving types of e-cigarette devices available on the market, including devices with adjustable power and deliveries, generating aerosol for testing is a challenge.

One study showed that there is a good correlation (about 74% of the time) between the cytotoxicity of an e-liquid and its aerosol (Behar et al., 2017). The authors suggested that developing a screening method in which e-liquids are first tested for toxicity followed by testing aerosols from those fluids that show toxicity would be more proactive, faster and less labour intense than producing aerosols for every sample. Sassano et al. found that aerosolisation did not increase nor changed the relative toxicity of e-liquids when assessed in HEK293T, HBECs and hASM cells, with the exception of one flavour, suggesting that this phenomenon is unique to that one flavour (Sassano et al., 2018). Rowell et al. found that the liquid and vapour forms of commercial e-liquids induced similar dose-dependent decrease in CALU3 cell viability and proliferation (Rowell et al., 2017).

4.5. CF menthol

CF Menthol had the lowest MEC values for the CF flavours in terms of cell cycle arrest, caspase 3/7, NF-κB activity, mitochondrial mass, GSH content and cellular ATP, however, these MECs were still 55–360 times higher than those seen for CSC (see Table 5). Menthol has a long history of use in a wide variety of applications, being generally regarded as safe (GRAS) in foods by the FDA. Menthol itself is not reported to be carcinogen, mutagenic or toxic to reproduction (Heck, 2010). However, menthol is known to act as increasing cellular permeability through the skin to compounds (Kitagawa and Li, 1999). The effects of menthol on cell cycle arrest have also been reported in prostate cancer cells (DU145) that over express the menthol receptor (TRMP8). Menthol is known to bind to this receptor, the activation of which is responsible for the cooling effect (Kennedy and Elliott, 1970). An MTT assay indicated that menthol significantly inhibited the cell growth of DU145 cells at 100 μM concentration. A cell cycle distribution and scratch assay analysis revealed that menthol induced cell cycle arrest at the G(0)/G(1) phase by the reduced expression of cell cycle checkpoint markers (CDKs) when analysed by western blot. Furthermore, menthol inhibited the migration of DU145 cells by down-regulating the focal-adhesion kinase (Wang et al., 2012).

This study has some limitations that need to be addressed in future studies. First, we tested e-liquids, not their aerosols. Future studies should aim at better reproducing human consumption, e.g., by

assessing and comparing the impact of repeated exposures to smoke and or aerosol over longer periods of time. However, the use of aerosol would also be more challenging in terms of exposure to the cells and the wide variety of potential devices and systems available on the market. Secondly, as described above, we used 24-h continuous exposures to e-liquids and CSC, a likely overestimate of human exposure.

4.6. Further work

Aligned with the 3R's strategies, an in vitro assessment framework should aim at obtaining a considerable level of accuracy in predicting the likelihood of adverse effects that may occur in humans from similar exposure conditions. Further dosimetry work is currently ongoing, exposing a 3D lung model at the air-liquid interface. This has the advantages of both exposing multiple cells types to e-liquid aerosol and to other potential constituents that may be generated at very low levels during vaping. However, 3D HCS is relatively still in its infancy due to the difficulty linked with 3D volumetric image analysis and potential differential effects being seen in the different cell types and at different cell depths. Future incorporation of methodology presented in this paper into the toxicity assessment framework will support a better prediction of any potential in vivo toxicity.

5. Conclusion

In this study, we used HCS to compare the biological effects of experimental and commercial e-liquids with 3R4F CSC in NHBE cells in a range of endpoints covering cellular health, energy production and oxidative stress. All tested e-liquids (with or without nicotine and flavourings) were typically non-cytotoxic and had no effect on most HCS endpoints at concentrations below 1%. The only e-liquid that impacted cell count (below 1% testing concentration) was CF Menthol, however the MEC value for this endpoint was 360 times higher than for 3R4F CSC. Our results clearly shows a lower toxicity of e-liquids, including flavoured e-liquids, when compared to CSC in this experimental setting. Typically, more than 100 times higher concentrations of CFs are required to elicit the same response as those as those observed for 3R4F CSC in specific endpoints.

While the presence of flavours in CFs triggered some effects (e.g. cell cycle arrest) not seen with base liquid formulation with or without nicotine, these effects were only observed at concentrations several orders of magnitude higher than CSC and at levels above which humans are likely to be exposed to, through daily usage. However, flavours play a critical role in attracting, and retaining smokers to e-cigarettes and are providing a viable alternative to combusted tobacco.

HCS has been shown to be a robust tool to screen e-liquids for mechanistic, multiparametric information on toxicity and presents a valuable tool for stewardship and prioritization purposes, when used as part of an overall Weight of Evidence approach (WoE) with other assays (including in vitro and clinical studies). We have undertaken further studies on e-cigarette aerosols using 3D organotypic models, offering more robust testing platform, to gain a better understanding of in vivo toxicity more closely recapitulating human exposure scenario and this work will be presented in a future publication.

Declaration of interests

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Transparency document

The Transparency document associated to this article can be found, in the online version.

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