A comparative in vitro toxicity assessment of electronic vaping product e-liquids and aerosols with tobacco cigarette smoke


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ABSTRACT

The use of electronic vaping products (EVPs) continues to increase worldwide among adult smokers in parallel with accumulating information on their potential toxicity and relative safety compared to tobacco smoke. At this time, in vitro assessments of many widely available EVPs are limited. In this study, an in vitro battery of established assays was used to examine the cytotoxic (Neutral red uptake), genotoxic (In vitro micronucleus) and mutagenic (Bacterial reverse mutation) responses of two commercial EVPs (blu GO™ disposable and blu PLUS™ rechargeable) when compared to smoke from a reference cigarette (3R4F). In total, 12 commercial products were tested as e-liquids and as aerosols. In addition, two experimental base liquids containing 1.2% and 2.4% nicotine were also assessed to determine the effect of flavour and nicotine on all three assays.

In the bacterial reverse mutation (Ames) and in vitro micronucleus (IVM) assays, exposures to e-liquids and EVP aerosols, with and without nicotine and in a range of flavourings, showed no mutagenic or genotoxic effects compared to tobacco smoke. The neutral red uptake (NRU) assay showed significantly reduced cytotoxicity (P < .05) for whole undiluted EVP aerosols compared to tobacco smoke, which by contrast was markedly cytotoxic even when diluted.

The reduced in vitro toxicological responses of the EVPs add to the increasing body of scientific weight-of-evidence supporting the role of high-quality EVPs as a harm reduction tool for adult smokers.

1. Introduction

According to Public Health England and the Royal College of Physicians, the use of electronic vaping products (EVPs) is likely to be at least 95% less harmful than smoking conventional cigarettes (McNeill et al., 2015; Royal College of Physicians, 2016). This view was recently reaffirmed, with a further comment from Public Health England suggesting that EVPs pose only a fraction of the harm that smoking does, and that adult smokers should be encouraged to switch (McNeill et al., 2018). Continuing to recognise that complete cessation of all tobacco and nicotine use as the best action smokers can take to improve their health, a growing number of international public health organisations, agencies and governments are clear that encouraging and assisting adult smokers, who are neither interested nor willing to quit smoking, to switch to nicotine products that are substantially less harmful than inhaled tobacco smoke is the next best option (https://www.tobaccocontrol.com/issue/1). Electronic vapour products are battery-powered devices that deliver aerosolised propylene glycol and/or glycerol and flavourings with or without nicotine to users. They do not contain tobacco or require combustion, but do simulate the visual, sensory, and behavioural aspects of smoking which conventional nicotine replacement therapy products do not. EVPs are available in many different configurations; the two principal distinctions being “open” systems which can be refilled by the consumer (e.g., tank or modular systems) or “closed” systems (e.g., replaceable cartridges pre-filled by manufacturers). When the user takes a puff, a heating element is activated converting the liquid in the cartridge into an aerosol that the user holds in the mouth or inhales.

In recent years, the worldwide use of EVPs has increased substantially with such products gaining acceptance with adult smokers as an alternative to tobacco cigarettes. As such, there is a need for a greater scientific understanding of the potential benefits and the potential toxicological impact, both inherent to EVPs and relative to tobacco cigarette smoke, for which EVPs seek to replace.

Tobacco smoke has been reported to contain many thousands of toxicants, including Harmful or Potentially Harmful Constituents...
associated with the tobacco combustion process (USFDA, 2012). These are associated with deleterious health effects including chronic obstructive pulmonary disease, cardiovascular disease and cancer. The types and concentrations of potential toxicants associated with EVPs is a topic of current scientific interest. Studies have indicated that EVP liquids and their aerosols contain fewer toxicants than tobacco smoke with many of the toxicants simply not present or are at levels equivalent to the tolerances permitted in medicinal products (Goniewicz et al., 2014; Tayyarah and Long, 2014). As a result, EVP liquids and their aerosols were expected to elicit markedly reduced biological responses in conventional regulatory in vitro toxicity assays when compared to tobacco smoke.

In the present study an in vitro battery of established assays was used to examine the mutagenicity (bacterial reverse mutation assay; Ames test), genotoxicity (in vitro micronucleus formation assay; IVM) and cytotoxicity (neutral red uptake assay; NRU) of e-liquids and their aerosols from two commercial closed device platforms (blu PLUS+™ and blu GO™). Using well-defined (ingredients and device specification) commercially available products from the USA, the primary focus of this study was to assess the biological effects of nicotine, flavour and device within each exposure (EVP aerosol or e-liquid) group. Due to the dosing regimen used, differences in toxicity between the same product delivered as an aerosol and as an e-liquid and based on a dose per dose comparison, was not undertaken. However, in all cases, comparisons were made between the effects of tobacco smoke and EVP aerosol exposure and positive controls, including nicotine and Sodium dodecyl Sulphate (SDS), and e-liquid exposure.

2. Materials and methods

2.1. Reagents

All reagents and equipment were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.2. Test products

All EVP products used were commercially available in the USA at the time of the study and contain a specified nicotine content as detailed by the manufacturer. The two EVP closed system devices tested were the blu disposable (blu GO™); volume, 1 mL; battery capacity, 345 mAh; coil resistance, 3.05 Ω; and the rechargeable (blu PLUS+™) consisting of two segments: a rechargeable battery section; battery capacity, 140 mAh; and a replaceable e-liquid cartridge (volume, 1.5 mL; coil resistance, 3.5 Ω) manufactured by Fontem Ventures B.V. (Netherlands). The e-liquids assessed in this study are reported in Table 1. Unflavoured base-liquids, containing propylene glycol and glycerol in 1:1 ratio, were obtained directly from Fontem Ventures B.V. (non-commercial). For comparison with EVP aerosols, the 3R4F reference cigarette was used (University of Kentucky, Kentucky, USA). Prior to smoking, 3R4F cigarettes were conditioned for at least 48 h according to the International Organisation of Standardisation standard (ISO: 3402, 1999). All e-liquids were stored at ambient room temperature until use.

2.3. Methods

2.3.1. Smoking and vaping regimes for tobacco smoke and EVP aerosol generation

For cytotoxicity and genotoxicity testing, EVP aerosol and 3R4F tobacco smoke were generated using a bespoke Smoke/Aerosol Exposure In vitro System (SAEIVS) designed in collaboration with Burghart Tabaktechnik, (Wedel, Germany). It is a versatile, high throughput system which can expose cells in 96 and 24 multi-well plates, the latter with transwell inserts (Fig. 1).

The SAEIVS is a five-port smoking/vaping machine directly connected to two independent exposure devices. A computer-controlled smoke dilution system allows the transfer of undiluted or diluted smoke/aerosol to the exposure devices. The initial dilution process is realised by mixing fresh tobacco smoke or EVP aerosol with a predefined volume of humidified, filtered air and is performed in a closed system of interconnected piston pumps. The two exposure chambers are supplied with the test tobacco smoke or EVP aerosol (55 mL over 2 or 3 s) by additional separate independent dilution systems that allow parallel exposures to occur at different dilution levels and at a rate of 78.6 mL/s. Tobacco smoke or EVP aerosol is delivered to the cells within 10 s to limit ageing effects and all wells of each plate are served with individual inlet and outlet ducts for exposure and extraction of each tobacco smoke or EVP aerosol dilution. In addition, after each smoke/aerosol exposure the cells are flushed with air (42.5 mL/s). After each step the smoke, aerosol and air are drawn away under vacuum. The use of a blanking plate in each exposure chamber enables puff-based dose response analyses to be performed. Furthermore, the separate chambers enable testing of the tobacco smoke or EVP aerosol from the same product in 2 independent in vitro assays and/or in different multi well plate formats at the same time. The system has been validated internally regarding delivery of tobacco smoke or EVP aerosol and the biological effects induced by the gaseous components by using appropriate positive controls. Also, the system can be easily set up for any required smoking/vaping regime.

For the mutagenicity assay (Ames), aerosol from EVPs and smoke from 3R4F were generated using a single port smoking machine, RM1 (Burghart Instruments, Wedel, Germany) connected to a three-port adapter, RM158. Ten millilitres of a bacterial suspension in phosphate buffered saline (PBS) was placed in an impinger and bubbled with freshly generated 3R4F tobacco smoke or EVP aerosol.

For each toxicological study, whole EVP aerosols were generated according to the vaping regime described by the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) recommended Method No.81 (CORESTA, 2015) (55 mL puff volume/3 s puff duration/30 s puff interval/square wave puff profile). Tobacco smoke was generated according to the Health Canada Intense smoking regime (55 ml

<table>
<thead>
<tr>
<th>Test product</th>
<th>Nicotine content (mg/mL)</th>
<th>Exposure matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base liquid</td>
<td>1.2</td>
<td>Blu GO™, Blu PLUS+™ √</td>
</tr>
<tr>
<td>Base liquid</td>
<td>2.4</td>
<td>Blu GO™, Blu PLUS+™ √</td>
</tr>
<tr>
<td>Classic tobacco</td>
<td>1.2</td>
<td>Blu PLUS+™ √</td>
</tr>
<tr>
<td>Classic tobacco</td>
<td>2.4</td>
<td>Blu GO™, Blu PLUS+™ √</td>
</tr>
<tr>
<td>Gold leaf</td>
<td>2.4</td>
<td>Blu PLUS+™ √</td>
</tr>
<tr>
<td>Magnificent menthol</td>
<td>1.2</td>
<td>Blu PLUS+™ √</td>
</tr>
<tr>
<td>Magnificent menthol</td>
<td>2.4</td>
<td>Blu GO™, Blu PLUS+™ √</td>
</tr>
<tr>
<td>Mint chocolate</td>
<td>2.4</td>
<td>Blu PLUS+™ √</td>
</tr>
<tr>
<td>Vivid vanilla</td>
<td>2.4</td>
<td>Blu GO™, Blu PLUS+™ √</td>
</tr>
<tr>
<td>Cherry crush</td>
<td>1.2</td>
<td>Blu PLUS+™ √</td>
</tr>
<tr>
<td>Cherry crush</td>
<td>2.4</td>
<td>Blu GO™, Blu PLUS+™ √</td>
</tr>
<tr>
<td>Caramel cafe</td>
<td>2.4</td>
<td>Blu PLUS+™ √</td>
</tr>
<tr>
<td>Strawberry mint</td>
<td>2.4</td>
<td>Blu PLUS+™ √</td>
</tr>
<tr>
<td>Berry cobbler</td>
<td>2.4</td>
<td>Blu PLUS+™ √</td>
</tr>
<tr>
<td>Blueberry high</td>
<td>2.4</td>
<td>Blu PLUS+™ √</td>
</tr>
<tr>
<td>Glacier mint</td>
<td>2.4</td>
<td>Blu GO™, Blu PLUS+™ √</td>
</tr>
<tr>
<td>Carolina bold</td>
<td>2.4</td>
<td>ND √</td>
</tr>
<tr>
<td>Carolina bold</td>
<td>2.0</td>
<td>Blu GO™, Blu PLUS+™ √</td>
</tr>
</tbody>
</table>

ND = Not determined. Blu GO™ is a disposable EVP and Blu PLUS+™ a refillable and rechargeable EVP. For EVP aerosol exposure studies one or both devices (blu PLUS+™ and blu GO™) were used. √ denotes that the e-liquids used are common to both the blu PLUS+™ and blu GO™ devices and were used for testing.
puff volume/2 s puff duration/30 s puff interval/bell shape puff profile). After 3 s exposure to cells in culture or bacterial suspensions in PBS, residual tobacco smoke and EVP aerosols were removed.

2.3.2. Cytotoxicity: Neutral Red Uptake Assay (NRU) with e-liquids, EVP aerosols and tobacco smoke

The *in vitro* NRU cytotoxicity assay (Borenfreund and Puerner, 1985) is widely used to determine the cytotoxicity of a variety of compounds. The cytotoxicity of e-liquids was measured using Hep-G2 cells (human hepatocellular carcinoma) and Beas-2b (human bronchial epithelium) cells. Since the relevant exposure to consumers is whole aerosol, it is more appropriate to make an assessment based on whole aerosol, rather than a fraction of the mainstream aerosol, i.e. the particulate phase (trapped as a condensate on a filter pad). Thus Beas-2b cells were raised to the air-liquid interface for exposure to EVP aerosol and tobacco smoke.

Beas-2b (European Collection of Authenticated Cell Cultures (ECACC)) cells were cultured in BEGM medium (BEGM supplemented with Lonza Bullet Kit, CC-3170) and Hep-G2 (American Type Culture Collection (ATCC)) were cultured in MIS medium (75% MEM Alpha medium and 25% Weymouth’s medium with 2 mM t-Glutamine, 4 mM Glutamax and Insulin-Transferrin-Selenium) supplemented with 1% serum substitute (Ultroser G; manufacturer). Prior to use, all stocks were stored frozen in liquid nitrogen and tested for the presence of mycoplasma contamination. Cell cultures were incubated at 37 °C, in humidified 95% air: 5% CO2.

For e-liquid exposures, 100 μL of Hep-G2 (2 × 10⁴/mL) or Beas-2b (0.8 × 10⁴/mL) cells in serum free medium were seeded into each of the inner 60 wells of a 96-well tissue culture plate and pre-incubated at 37 °C, 5% CO₂ for 20 ± 3 h. Following removal of the culture medium, the cells were exposed to 200 μL of increasing concentrations of e-liquids (ranging between 0 and 5 mg/mL) for 65 ± 2 h. In order to avoid the cross contamination of wells from volatile chemical components from e-liquids, in the NRU assay, and aerosol, in both the NRU and IVM assays, each plate was sealed with CO₂ permeable plastic film during incubation.

For EVP aerosol and 3R4F smoke exposures, each well of a 96 multi-well round bottom plate was initially filled with 25 μL of Collagen I solution (20%, PureCol® EZ Gel; 2%, 1 M HEPES buffer; and 78% of BEGM medium) and incubated at 37 °C, in humidified 95% air: 5% CO₂ for 18–24 h. Following liquid removal, 100 μL of Beas-2b (0.5 × 10⁴/mL) cells in serum free medium were added into each of the inner 60 wells of the 96 multi-well plates and pre-incubated at 37 °C, 5% CO₂ for 20 ± 3 h. Directly before 3R4F smoke and EVP aerosol exposure, the culture medium was removed by suction and reverse plate centrifuged (10 g for 10 s), which has previously been observed to have a no effect on cell viability following exposure to 400 puffs of air (data not shown). The plates were then placed in the SAEVS exposure chamber and exposed to diluted whole tobacco smoke (0–11 puffs at 1:8 to 1:14 dilutions) or undiluted EVP aerosol (0–100 puffs) according to the smoking and vaping regimes described in Section 2.3.1. Sodium dodecyl sulphate (0.0–15 μg/mL) and nicotine (0.0–1.0 mg/mL) were used as positive controls for e-liquid and 3R4F smoke for EVP aerosol exposure. Following exposure, 200 μL of fresh culture medium was added to each well and the cells incubated at 37 °C, in humidified 95% air, 5% CO₂ for a further 65 ± 2 h. Following incubation, the culture medium was removed and replaced with 200 μL of neutral red staining solution in culture medium (supplemented with 20 mM HEPES and 10% FBS) and incubated at 37 °C, 5% CO₂ for 3 h. After staining, the cells were washed once with 150 μL of 1.34% Calcium Chloride and then lysed with 100 μL of Ethanol/acetic acid solution (1% Glacial acetic acid and 50% in water) over 30 min at room temperature and pressure (RTP). The neutral red, which is retained in the lysosomes of viable cells, was released and quantified by measuring the absorbance at 540 nm on a microplate reader (TECAN Sunrise).

All exposures were conducted in triplicate in a minimum of two independent experiments. A nonlinear four parameter logistic plot was applied to the data and the concentration of e-liquid (mg/mL) or EVP
aerosol and 3R4F smoke (puff number) that resulted in 20% cytotoxicity (EC<sub>20</sub>) were calculated.

Significant differences (p < .05) between nicotine, SDS, base liquids and the flavoured products (comparisons were made between equivalent 1.2% and 2.4% nicotine base liquids) were determined using a one-way analysis of variance (ANOVA) with Dunnett’s post hoc multiple comparison test. A two-way ANOVA was also conducted on the e-liquid data to determine the effect of nicotine (1.2% nicotine vs 2.4% nicotine for base liquid, classical tobacco, magnificent menthol and cherry crush; n = 4), and cell type (all e-liquids used for each cell culture; n = 18). The effect of nicotine using EVP aerosols (1.2% nicotine vs 2.4% nicotine for base liquid, classical tobacco, magnificent menthol and cherry crush; n = 4) and device (blu® GO™ vs blu PLUS+™; n = 8) on cytotoxicity was also assessed.

2.3.3. Genotoxicity: In vitro Micronucleus Assay (IVM) with EVP e-liquid, aerosol and tobacco smoke
The in vitro micronucleus assay is a genotoxicity test for the detection of cytoplasmic micronuclei (MN) of interphase cells and as recommended by the OECD (OECD guideline no. 487). Micronuclei may originate from either acentric chromosome fragments (i.e. lacking a centromere), or whole chromosomes that are unable to migrate to the poles during the anaphase stage of cell division. The assay detects the activity of clastogenic and aneugenic chemicals in cells that have undergone cell division during or after exposure to the test substance.

Aroclor 1254 induced rat liver microsomal fraction S9 (Lot No. MolTox S9 3604), obtained from BioSepra S.A., was stored frozen at −70 °C until use. Human lymphoblastoid TK6 cells (Cell Lines Services, Eppelheim, Germany) ± S9 were used to assess the genotoxic potential of e-liquids, and Chinese Hamster lung fibroblast V79 cells (European Collection of Authenticated Cell Cultures (ECACC)) for EVP aerosols and 3R4F smoke in the presence of S9. Prior to use, all stocks were stored frozen in liquid nitrogen and tested for the presence of mycoplasma contamination.

For all e-liquids, an initial concentration of 16 mg/mL (in culture medium) was prepared and diluted to a range of working concentrations (1.25–5.0 mg/mL). Positive controls (Table 2) and e-liquids were diluted in RPMI medium supplemented with 10% heat inactivated horse serum for “long-term treatment” (LT+S9) and “short-term treatment” (ST+S9) or without serum for “short-term treatment” (ST-S9), all containing 1% antibiotic / antimycotic solution.

For e-liquid long-term treatment, 1 mL of a TK6 cell suspension (1 × 10<sup>6</sup> cells/mL) in RPMI cell culture medium (supplemented with 10% heat inactivated horse serum and 1% antibiotics/anti-mycotics (AM)) was added to each well of a 24 well plate and incubated overnight at 37 °C, in humidified 95% air: 5% CO<sub>2</sub>. One millilitre of a twofold concentration of each e-liquid (prepared with 10% heat inactivated horse serum and 1% AM) was applied to each well of the plate at 4 dose levels (n = 4/dose). Positive and negative controls were applied concurrently (n = 4/dose). A 125 μL cell suspension per well from each dose group was pooled to determine the cell number at the beginning of the experiment using the Vi-cell cell counter device (Beckman Coulter). The plates were then incubated at 37 °C, 5% CO<sub>2</sub> for 4 ± 4 h.

For the short-term treatment with and without S9, 0.5 mL of TK6 (1.5 × 10<sup>6</sup> cells/mL) cells in supplemented RPMI medium (containing 2% inactivated horse serum and 1% AM stock solution) were incubated overnight in each well of a 24 well plate at 37 °C in humidified 95% air: 5% CO<sub>2</sub>. The following day, pre-prepared e-liquids diluted in RPMI culture medium with and without S9 mix (13% with 10% S9) and at 1.3-fold target concentration, were applied (1.5 mL) to each well at 4 dose levels (n = 4/dose), resulting in a 1 × final concentration per well. Following an incubation of 4 h the cells of each of the four replicates were pooled and centrifuged at 600 g for 5 min. The cell pellet was then re-suspended in 9 mL of RPMI culture medium (containing 10% heat inactivated horse serum + 1% AM) and reseeded into fresh 24 well plates at 2 mL per well (n = 4/dose). The cells were incubated at 37 °C, in humidified 95% air, 5% CO<sub>2</sub> for 40 ± 4 h. The remaining cell suspension was used to determine the cell number using a Vi-cell cell counter and before the recovery period.

For EVP aerosol and 3R4F tobacco smoke exposures, 24 multi-well plates were filled with 250 μL/well of Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Membrane inserts (0.4 μm pore size) were then placed in each well and filled with 400 μL of 1 × 10<sup>6</sup> V79 cells per mL. The plates were incubated at 37 °C, in humidified 95% air, 5% CO<sub>2</sub> for 18 ± 2 h. Following incubation, the culture medium covering the cells was removed and the inserts transferred into new 24 well plates containing 250 μL HEPES buffer (20 mM) and exposed to undiluted whole EVP aerosol (0–100 puffs) or 12 puffs of 3R4F diluted tobacco smoke (1:4 and 1:5 dilution) according to the protocol described in Section 2.3.1. During exposures, no culture medium was present in the upper compartment of the inserts allowing direct contact of the EVP aerosol or tobacco smoke with the cells. Following exposures, the inserts were transferred to fresh 24 well plates containing 250 μL DMEM. 300 μL of culture medium containing 10% S9 mix (with 10% S9 fraction) was then carefully added to each insert. Following 3 h incubation at 37 °C, in 95% humidified air, 5% CO<sub>2</sub> the apical S9 mix was removed and the cells covered with DMEM containing 10% FCS. The cells were then incubated for a further 20 ± 2 h to allow at least one cell division cycle. Identically pre-grown cells were detached from control inserts with detachment solution (accutase) and counted using a hand-held cell counter (Scepter cell counter, Millipore) to determine cell number before the recovery period.

After the incubation period, V79 cells exposed to EVP aerosols and 3R4F tobacco smoke were again detached with cell detachment solution (accutase) and counted using the handheld cell counter. Suspensions of TK6 cells exposed to e-liquids were counted using a Vi-cell counter.

Cell suspensions were exposed to 37.5 mM KCl for 5 min and then spun for 5 min and fixed onto slides using a cytopsin at 590 g (TK6 cells) and 380 g (V79 cells). The supernatant was removed, and the preparations were dried by repeated centrifugation. The cells were then chemically fixed (Methanol [150 mL]/glacial acetic acid [18.5 mL]/37% formaldehyde [1 mL]/water [30.5 mL]) onto the slides, washed once in methanol and then air dried. DNA-containing structures were stained with 1 μg/mL of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) in mounting medium (Vectorshied H-1000). The numbers of nuclei were determined microscopically using the Metafer imaging system coupled to a fully automated Microscope (Imager, Z2, Zeiss) in more than 1000 interphase cells per dose level (two replicates for V79; four for TK6 cells), negative and positive controls.

As proposed by Fenech (1993), the criteria for analysing micronuclei included; 1. Did the cytoplasm remained intact?, 2. Were any micronuclei present separated in the cytoplasm or just touching the main nucleus?, 3. Were the main nuclei of the cells of approximate equal size? 4. Was the diameter of the micronucleus smaller than 1/3 of the main nucleus? For consideration as a positive IVM response, there needed to be a reproducible dose dependent increase in micronuclei frequency and the increased frequency, at any dose, must be significantly different to that of the negative control. Cytotoxicity was
calculated as relative population doubling (RPD, OECD 487).

An initial linear regression analysis was performed to determine the effect of dose on micronucleus frequency (i.e. the fold change of percentage micronucleus at each dose, relative to the vehicle control) following e-liquid, EVP aerosol and 3R4F smoke exposures. A one-way ANOVA with a Dunnett’s post hoc comparison test was conducted on e-liquids and a pair-wise chi-square comparison on the EVP aerosol and 3R4F smoke exposed cells to determine the effect of individual dose on micronucleus frequency when compared to their corresponding vehicle control. A p < .05 was considered statistically significant.

A three-way ANOVA was also conducted on the blu PLUS+™ data following e-liquid exposure, to determine whether there was a cytotoxic or micronuclear frequency effect due to; 1. nicotine, dose or S9 treatment (ST + S9 vs ST-S9) and 2. nicotine, dose or treatment length (ST-S9 vs LT-S9). In addition, a 2-way ANOVA was also conducted following EVP aerosol exposure to assess the effect of dose and nicotine concentration (blu PLUS+™ products only) and the effect of dose and device (blu PLUS+™ vs blu GO™) on cytotoxicity and micronuclear frequency.

2.3.4. Mutagenicity: Bacterial Reverse Mutation Assay (Ames) with e-liquid, undiluted EVP aerosol or diluted tobacco smoke

The Ames assay (bacterial reverse mutation assay) is widely used as an initial screen to determine the mutagenic potential of chemical compounds with a high predictive correlation with in vivo carcinogenicity (Maron and Ames, 1983; Thorne et al., 2015). The Ames reverse mutation assay was performed as described by Maron and Ames, 1983 and in general accordance to the Organization for Economic Co-operation and Development, OECD, guideline No. 471 using the bacterial strains reported in Table 3. The induction of reverse mutations with each e-liquid was tested with five bacterial strains (S. typhimurium TA98, TA100, TA102, TA1535 and TA1537) Biochem GmbH Giessen, Germany, Trinova) with and without S9. For EVP aerosol exposure, S. typhimurium TA100 with S9 treatment was used. All stocks were frozen at −70°C prior to use. Each concentration of test e-liquid, EVP aerosol, 3R4F smoke, negative (vehicle) and positive controls were tested in triplicate. Six replicate readings were conducted for the vehicle controls.

For e-liquid testing, 30 mL of Nutrient Broth No.2 (OXOID) culture for each bacterial strain was prepared in a 100 mL Erlenmeyer flask with one bacterium-coated CRYO-glass bead (TA98, TA100, TA102, TA1535 and TA1537). For TA98, TA100 and TA102 ampicillin was added (final concentration, 25 μg/mL), for TA102 tetracycline was added (final concentration, 2 μg/mL) and no antibiotic was added to TA1535 and TA1537. Fifty microlitres of a 1:10 dilution of e-liquid in water (1–5 μg/plate) together with 100 μL of bacterial culture were then added to 0.5 mL of an S9 mix (+S9) or buffer (0.2 M sodium phosphate pH 7.4 (-S9)). After 20 min incubation at 37°C the tubes were mixed with 2 mL of Top Agar (45°C) and then poured over Vogel-Bonner (VB)-agar plates, which were rotated and tilted to distribute the top agar evenly over the plates. When the top agar solidified, the plates were then inverted and incubated at 37°C for 48–72 h.

The use of particulate phase only in such biological test fails to account for any potential toxicity effects due to the low molecular weight compounds in the gas or aerosol phase. Thus, EVP aerosol and 3R4F tobacco smoke smoke exposures were performed using the micro-suspension assay procedure. Briefly, bacterial cultures (four flasks of 30 mL) were started with 0.4 mL of a 6-h pre-culture of TA100 followed by overnight incubation and shaking at 120 rpm. The cultures were then centrifuged to obtain a 10-fold concentrated bacteria suspension (Kado et al., 1983). After overnight incubation the 120 mL of bacterial suspension were then centrifuged (1800 g for 15 min) and the pellet resuspended in 12 mL Ca²⁺ and Mg²⁺ free Dulbecco’s PBS. A 10 mL suspension of TA100 bacterial suspension in PBS was added to an imit-inger through which EVP aerosol (60–300 puffs) or 3R4F tobacco smoke (1–5 cigarettes: 10–50 puffs) was bubbled. Fifty microlitres of exposed bacterial culture was then mixed with 0.5 mL of S9 and then poured over VB-agar plates. After 2 days of incubation, the number of revertant colonies growing on the plates was counted. All colonies were counted with a Synbiosis ProtoCOL SR automated colony counter, (Frederick, MD, USA) to determine the number of bacterial revertants/plate.

Assay acceptance criteria included the mean negative control colony count falling within the normal historical range, positive controls inducing a clear increase in revertant numbers, an active S9 preparation and no more than 5% of the plates lost through contamination or some other unforeseen event. The sample was considered mutagenic if; 1. The assay produced a two-fold increase or greater in the number of induced revertants when compared to the negative control (Vehicle, or vehicle plus S9 mix) in strains TA98, TA100 or TA102 and a three-fold increase or greater in strains TA1535 or TA1537, 2. A positive linear dose-fold increase in revertants response and 3. A reproducible positive control response. Assays were repeated for confirmation where positive and the acceptance criteria is in accordance with previously published studies (Le Godec et al., 2019).

Mutagenic activity was calculated from the slope of the dose-response (fold increase in revertants) curve (non-threshold model) with differences in the response following product exposure and the solvent controls tested for significance (p < .05) using a one-way ANOVA and Dunnett's post hoc test. A three-way ANOVA was conducted on the dose-fold increase in revertant numbers for each Salmonella typhimurium strain to determine the effect of dose, treatment and nicotine content of the e-liquid preparations. A two-way ANOVA was also conducted on the dose-fold increase in revertant numbers following exposure to EVP aerosol to determine; 1. The effect of dose and nicotine and 2. The effect of dose and device (blu PLUS+™ vs blu GO™).

2.4. Statistical analyses

In all cases statistical analyses were performed using GraphPad Prism version 8.0 software.

Table 3
Salmonella typhimurium strains characteristics, source and positive controls.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>Antibiotic resistance</th>
<th>Positive controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without S9 (μg/plate)</td>
<td>With S9 (μg/plate)</td>
</tr>
<tr>
<td>TA98a</td>
<td>His D3052 (frameshift)</td>
<td>Ampicillin</td>
<td>2-Nitrofluorene (4 μg/plate)</td>
</tr>
<tr>
<td>TA100c</td>
<td>His G46 (Base pair substitution)</td>
<td>Ampicillin</td>
<td>NaAzide (1 μg/plate)</td>
</tr>
<tr>
<td>TA102</td>
<td>His G428 (Base pair substitution)</td>
<td>Ampicillin, Tetracycline</td>
<td>Mitomycin C (1 μg/plate)</td>
</tr>
<tr>
<td>TA1535b</td>
<td>His H46 (Base pair substitution)</td>
<td>None</td>
<td>NaAzide (1 μg/plate)</td>
</tr>
<tr>
<td>TA1537b</td>
<td>His C3076 (Frameshift)</td>
<td>None</td>
<td>9-Aminoacridine (50 μg/plate)</td>
</tr>
</tbody>
</table>

Abbreviations: His = Histidine.

a National Collection of Type Cultures, Porton Down, Salisbury, UK.

b Covance Laboratories Inc., USA.

c Professor Ames’ laboratory.
3. Results

3.1. Cytotoxicity of e-liquids, EVP aerosol and tobacco smoke

To determine the cytotoxicity of e-liquids (Beas-2b and Hep-G2 cells), whole undiluted EVP aerosols and tobacco smoke (Beas-2b cells only) were measured based on the concentration (mg/ml) or puff number that resulted in a 20% reduction in cell viability (EC_{20}), as evaluated by the uptake of neutral red (Fig. 2). The EC_{20} values are reported in Table 4.

For positive controls, the EC_{20} for SDS was significantly lower than the EC_{20} for all 17 blu PLUS+™ e-liquid products and regardless of cell type. For nicotine, the EC_{20} was significantly lower in 50% (9/18) and 89% (16/18) of all e-liquids tested when Beas-2b and Hep-G2 cell cultures were used respectively. As expected, the number of puffs required to induce a 20% reduction in cell viability following tobacco smoke exposure to Beas-2b cells was significantly (p < .05) lower than the number of puffs, that induced a similar degree of toxicity, ranging between 143 and 1191 times more toxic than any of the flavoured or unflavoured EVP aerosols generated from either device (Table 4).

To determine the potential cytotoxic effect of flavours, all flavoured e-liquids and aerosols were compared with their equivalent base liquid control. The concentration of base liquid that resulted in 20% cell viability (EC_{20}) was significantly higher, 3 of 5 EC_{20} values from EVP aerosols generated from the blu GO™ device were significantly (p < .05) more cytotoxic than their equivalent base liquid controls.

On an assessment of the effect of nicotine on cytotoxicity following e-liquid and EVP aerosol exposure, similarly flavoured products (Base liquid, Classic Tobacco, Magnificent Menthol and Cherry Crush) were compared at 1.2% and 2.4% nicotine. For e-liquids, Beas-2b cells were 31% (p < .005) and Hep-G2 cells 28% (p < .05) more sensitive to 2.4% nicotine than 1.2% nicotine (Fig. 3A). EVP aerosols generated from the 2.4% nicotine products were also significantly more cytotoxic than aerosols generated from similarly flavoured products containing 1.2% nicotine (Fig. 4A).

When all e-liquid cytotoxicity data from each cell culture (n = 17) were compared, Hep-G2 cells were found to be 30% (p < .0001) more sensitive to exposure than Beas-2b cells (Fig. 3B). In addition, on comparison of the cytotoxic effects of aerosols generated from the blu PLUS+™ and blu GO™ devices, the EVP aerosol from the blu GO™ device was statistically (p < .0005) more cytotoxic than the aerosol generated from the blu PLUS+™ device (Fig. 4B). As with comparisons made between EVP aerosols containing 1.2% and 2.4% nicotine, both devices generated aerosols that were on average between 143 and 1191-fold less cytotoxic than that observed following cigarette smoke exposure.

Overall, nicotine (e-liquids and EVP aerosol), cell type and flavourings (e-liquids) and device (blu PLUS+™ vs blu GO™) showed significant effects on cytotoxicity and although EVP aerosols generated a cytotoxic response in these mammalian cells under the conditions of test, these responses were substantially less cytotoxic than that following tobacco smoke exposure.

3.2. Genotoxicity of e-liquids, EVP aerosol and tobacco smoke

The aneugenic and clastogenic potential of e-liquids and whole EVP aerosols was assessed through their effects on the chromosomes of human lymphoblastoid TK6 and Chinese hamster lung V79 cells. An increase in the formation of MN is considered an early marker for the identification of potential carcinogenesis (Bonassi et al., 2011).

In all studies, all genotoxic positive controls (Figs. 5A, 5B, 6A and 6B) and exposure to tobacco smoke (Fig. 5B and 6B) induced statistically significant (p < .05) increases in cytotoxicity (Relative population doubling) and micronucleus frequency when compared to vehicle and regardless of exposure matrix.

No effect of dose, nicotine concentration, S9 treatment (ST + S9 vs ST-S9) or length of exposure (ST + S9 vs LT + S9) was observed on cytotoxicity (RPD) following exposure of TK6 cells to e-liquids. Although exposure of V79 cells to EVP aerosol induced a significant (p < .0001) dose dependent increase in cytotoxicity (Fig. 7) no significant difference between products or any effect of nicotine was observed.

The effect of e-liquid and EVP aerosol exposure on the MN dose-response is shown in Table 5. Only 6 products demonstrated a significant micronucleus dose-response, all of which were e-liquids. Three of these products had at least one concentration that was significantly different from the zero control. However, except for base liquid 2.4% nicotine, two of these three e-liquid products exhibited negative slopes. As the MN frequencies for all base liquid 2.4% nicotine concentrations were below the zero control, no significant effect on MN induction was therefore observed following any e-liquid or EVP aerosol exposure.

3.3. Mutagenicity of e-liquids, EVP aerosol and tobacco smoke

The mutagenic activity of e-liquids, undiluted EVP aerosol and diluted 3R4F tobacco smoke was assessed using the Ames assay. The slope
of the dose–response curve was determined for each product and used to derive the mutagenic potency (number of mutants per unit concentration of product tested). The fold-increase in potency was statistically compared with the vehicle response.

All e-liquid positive controls induced significantly increased revertant numbers (> 3-fold), regardless of S9 treatment (Fig. 8A). In all but one strain (TA102), S9 treatment resulted in higher revertant numbers than without S9. For 3R4F smoke exposure there was a significant dose (puff) dependent increase in revertant numbers (>3-fold), regardless of S9 treatment (Fig. 8A). In all 8 samples tested when generated using the blu GO™ device.

The dose response effect of e-liquid exposure on revertant numbers (slope) with and without S9 is shown in Table 6. No significant effect of dose, S9 treatment nor nicotine concentration was observed following e-liquid exposure using the five bluPLUS™ and bluGO™ devices. The same number of samples were assessed for aerosol toxicity (except Carolina Bold 2.4% nicotine) when generated from the blu PLUS+™ device and 8 samples tested when generated using the blu GO™ device.

The standard toxicological battery of tests used in the current study, included the NRU assay to assess cytotoxicity (Borenfreund et al., 1985), the in vitro micronucleus assay to measure mammalian genotoxicity (OECD, 2016. Test No. 487) and the bacterial reverse mutation (Ames) assay to determine mutagenicity (OECD, 1997, Test No. 471). These form part of a battery of recommended assays (ICH, 2011; COM, 2011; CORESTA, 2004) that are used for product assessment, regulatory applications and prior to more in depth and mechanistically informative in vitro assays. Although, for e-liquids the recommended procedures were followed, where appropriate, modifications were included to determine the direct effects of EVP aerosol and 3R4F smoke on cytotoxicity and in vitro micronucleus. Such modifications have been used previously (Li, 2016; Leigh et al., 2016; Wan et al., 2009) since the exposure of interest to consumers is the effect from whole EVP aerosol. For comparative purposes, the cytotoxic response was calculated as the EC20 as it was not possible to derive the EC50 (concentration of product that kills 50% of the cells) or higher for all products due to the low cytotoxic response following e-liquid and EVP aerosol exposure. Additionally, the potential adverse effects associated with hyperosmotic shock (Gonzalez-Suarez et al., 2017; Czekala et al., 2019) at high e-liquid doses required to generate the EC20 was also considered.

Twelve different flavoured (Table 1) e-liquids, two non-flavoured base liquid formulations (containing 1.2% and 2.4% nicotine), two positive controls (nicotine and SDS) and 3R4F tobacco smoke were assessed for cytotoxicity in two cell culture systems, one lung derived and exposed to both e-liquid and EVP aerosol (Beas-2b), and one liver derived and exposed to e-liquid only (Hep-G2). Exposure to the nicotine positive control was toxic to both cell cultures and more toxic than most of the e-liquids tested. Although this acute toxic response to nicotine

4. Discussion

This in vitro comparative toxicological study was designed to evaluate both e-liquids and aerosols generated from two closed-system EVP devices and in comparison, to the response following exposure to positive controls for e-liquid products and cigarette smoke for EVP aerosols. For the regulatory toxicity assays (Ames, IVM and neutral red), a total of 12 commercial e-liquid flavours with 4 products and two base liquid formulations containing 1.2% and 2.4% nicotine, were tested using both the blu PLUS+™and blu GO™ devices. The same number of samples were assessed for aerosol toxicity (except Carolina Bold 2.4% nicotine) when generated from the blu PLUS+™ device and 8 samples tested when generated using the blu GO™ device.

Table 4

<table>
<thead>
<tr>
<th>Test material</th>
<th>Nicotine (%)</th>
<th>EC20[mg/mL]</th>
<th>EC20[puffs]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>blu PLUS+™</td>
<td>blu GO™</td>
</tr>
<tr>
<td>Nicotine 100</td>
<td>1.2%</td>
<td>0.05 ± 0.01b</td>
<td>0.035 ± 0.015b</td>
</tr>
<tr>
<td>SDS 0.0</td>
<td>2.3%</td>
<td>0.002 ± 0.0003</td>
<td>0.003 ± 0.0003</td>
</tr>
<tr>
<td>Base liquid 1.2</td>
<td>1.44 ± 0.07***</td>
<td>1.54 ± 0.37</td>
<td>59.49 ± 5.58</td>
</tr>
<tr>
<td>Base liquid 2.4</td>
<td>0.39 ± 0.13***</td>
<td>1.18 ± 0.11</td>
<td>90.51 ± 39.75</td>
</tr>
<tr>
<td>Classic tobacco 1.2</td>
<td>0.39 ± 0.13***</td>
<td>1.18 ± 0.11</td>
<td>90.51 ± 39.75</td>
</tr>
<tr>
<td>Classic tobacco 2.4</td>
<td>0.95 ± 0.16**</td>
<td>0.49 ± 0.14′′</td>
<td>93.47 ± 13.19</td>
</tr>
<tr>
<td>Gold leaf 2.4</td>
<td>0.40 ± 0.12***</td>
<td>0.82 ± 0.22′′</td>
<td>87.43 ± 15.34</td>
</tr>
<tr>
<td>Magnificent menthol</td>
<td>0.38 ± 0.21****</td>
<td>0.57 ± 0.13′′</td>
<td>143.75 ± 79.86″</td>
</tr>
<tr>
<td>Mint chocolate 2.4</td>
<td>0.92 ± 0.14***</td>
<td>0.77 ± 0.29″</td>
<td>51.89 ± 22.03</td>
</tr>
<tr>
<td>Blueberry high 2.4</td>
<td>0.21 ± 0.35****</td>
<td>0.98 ± 0.25</td>
<td>153.00 ± 36.19</td>
</tr>
<tr>
<td>Cherry crush 1.2</td>
<td>0.72 ± 0.39***</td>
<td>1.76 ± 0.13</td>
<td>103.00 ± 16.86</td>
</tr>
<tr>
<td>Cherry crush 2.4</td>
<td>0.64 ± 0.12′′</td>
<td>1.07 ± 0.23</td>
<td>51.21 ± 36.19</td>
</tr>
<tr>
<td>Caramel café 2.4</td>
<td>0.21 ± 0.06***</td>
<td>0.94 ± 0.04′</td>
<td>38.49 ± 20.62</td>
</tr>
<tr>
<td>Carolina bold 2.4</td>
<td>1.20 ± 0.11</td>
<td>1.00 ± 0.07</td>
<td>51.08 ± 32.55</td>
</tr>
<tr>
<td>Berry cobbler 2.4</td>
<td>0.18 ± 0.06***</td>
<td>0.70 ± 0.08**</td>
<td>95.05 ± 22.10</td>
</tr>
<tr>
<td>Blueberry high 2.4</td>
<td>0.37 ± 0.11***</td>
<td>0.84 ± 0.26′′</td>
<td>93.47 ± 13.19</td>
</tr>
<tr>
<td>Glacier mint 2.4</td>
<td>0.50 ± 0.10***</td>
<td>0.81 ± 0.13″</td>
<td>39.88 ± 8.68′′</td>
</tr>
<tr>
<td>Strawberry mint 2.4</td>
<td>0.79 ± 0.20″</td>
<td>0.73 ± 0.01″</td>
<td>95.05 ± 22.10</td>
</tr>
</tbody>
</table>

Data represents the mean ± SD (n = 3–6). ****p < .001, ***p < .005 ** p < .01, * p < .05 compared to equivalent nicotine base liquid control.

b p < .05 for all blu PLUS+™ e-liquid products assessed in Beas-2b cells except for Classic Tobacco (1.2% and 2.4%), Magnificent Menthol (1.2% and 2.4%), Vanilla 2.4%, Caramel Café 2.4%, Berry Coblher 2.4%, Blueberry 2.4% and Glacier Mint 2.4%.

p < .05 compared to all e-liquid products.

p < .05 compared to all devices generating aerosol.
has been observed previously (Vlasceanu et al., 2018; Moga et al., 2016; Chen et al., 2015; Bahl et al., 2012), the sub-toxic, chronic exposure to nicotine is known to increase cell viability (Stabile et al., 2018). This duality in response must be carefully considered when addressing the subject of toxicity of these products, especially when nicotine exposure in vapers may more closely represent a repeated and subtoxic exposure.

Exposure to e-liquids induced a dose dependent increase in cytotoxicity, which was not wholly driven by the acute effects of nicotine exposure, with nicotine contributing to 3.5%–13.5% of the cytotoxicity following exposure to Hep-G2 cells and 1.5%–7.5% for Beas-2b cells. Hep-G2 cells were also found to be 30% more sensitive to the effect of e-liquid exposure than Beas-2b cells, the reason for which is unclear. However, different cell types have differing sensitivities (Scheffler et al., 2015; Li, 2016) to a toxic insult which may be due, in part, to the robustness of the cell system. Passage number, cell density and, specifically for Beas-2b cells being raised to the air-liquid interface, are robustness of the cell system. Passage number, cell density and, specifically for Beas-2b cells being raised to the air-liquid interface, are adaptive and cellular changes that may impact the response of Beas-2b cells when at the air-liquid interface just prior to exposure. In the current study, the primary focus was also to address the effects of device, flavours and nicotine on the cellular response following exposure. The experimental dosing regimen did not allow for comparative assessments to be made, on a dose per dose basis, on the same products delivered as an aerosol and directly as an e-liquid. However, all cell culture responses were compared to combustible cigarette smoke for EVP aerosol exposures and a variety of experimental controls, including nicotine and SDS, for e-liquid exposures. Both cell cultures showed good dose response relationships with nicotine, SDS and 3R4F smoke and were considered appropriate for toxicity comparisons (Li, 2016) within this study.

Flavours have been shown to play a critical role in attracting – and retaining – adult smokers to EVPs thereby directly contributing to tobacco harm reduction (Farsalinos et al., 2013; Biener and Hargraves, 2014). Flavoured e-liquids tended to be more biologically active to both cell cultures than equivalent base liquid formulations and is in line with published data (Leigh et al., 2016; Omaiye et al., 2019; Czekala et al., 2019). However, this was not mirrored following aerosol exposure. This lack of concordance between e-liquid and aerosol is unclear, but may be due to many potential effects, including changes in the cellular physiology of the Beas-2b cells that may occur during the short period in which these cells are adapting to exposure at the air-liquid interface (Briske-Anderson et al., 1997) or that individual flavour constituents may not be aerosolised in the same ratio to PG and VG as that found in the e-liquid. Additionally, the dose of e-liquid delivered to the cells is significantly greater than that delivered by aerosol and any differences between products and their base formulation may only become visible at these higher concentrations. Therefore, further studies are required to allow more accurate comparisons of toxicity to be made within and between differently delivered formulations. Aerosol generated from blu GO™ was also significantly more active than aerosol generated from blu.
PLUS+™. The blu GO™ operates at a much higher power level than blu PLUS+™ and although this may generate larger puff volumes, which has not been determined experimentally to date, and allow the delivery of higher doses per puff, to the user, than the blu PLUS+™ device, the differences seen in this study are likely due to changes in the chemical nature of the aerosol as cells were exposed to the same set volume (55 ml) of aerosol. On comparison to smoke generated from 3R4F, the toxicity of EVP aerosol, generated from either device and at both 1.2% and 2.4% nicotine, was between 143 and 1191 times less toxic. However, the variation in aerosol toxicity across the samples, using the blu PLUS™ device and Beas-2b cells was much lower than that observed for the equivalent e-liquids (3.3-fold compared to 4.6-fold respectively). Additionally, the rank order of toxicity was also different between equivalent EVP aerosols and e-liquids, potentially indicating that the way in which the delivery of e-liquid formulations, to cells in culture, is achieved may have an impact on the toxicity of the product under investigation. Further characterisation of the chemical profile of the aerosol from the two devices and toxicity comparisons at equivalently delivered doses will be required to clarify these observations.

TK6 and V79 cells were used to assess the induction of in vitro micronucleus following exposure to e-liquids, EVP aerosols and tobacco smoke. TK6 human lymphoblastoid cells are derived from p53 competent human peripheral blood lymphocytes and are therefore considered a more appropriate choice of cells for MN analysis (Fowler et al., 2014). The rodent cell line V79 was used in the assessment of the EVP aerosol from both devices and although this cell line is OECD approved, it is not p53 competent. The possible role of p53 in the IVM response has been addressed previously (Fowler et al., 2012; Whitwell et al., 2015; Thorne et al., 2019). Although differences between p53 competent and non-competent cell lines with respect to DNA damage and repair are apparent, the use of both V79 and TK6 cells in this study were deemed acceptable due to the ability to distinguish between tobacco smoke, EVP aerosols and e-liquids. However, V79 cells are of lung origin, and adherent (TK6 cells are grown as suspended cultures) and therefore considered important for culturing at the air-liquid interface and for aerosol exposure. In the current study exposure to tobacco smoke induced a significant increase in the number of micronuclei, an observation confirmed in a variety of published studies (Crooks et al., 2013; Combes et al., 2013; Takahashi et al., 2018).
Although exposure of cells to e-liquids had no significant effect on cytotoxicity, there was a significant effect of nicotine on micronucleus induction. This is in concordance with previously published studies on the direct genotoxic effect of nicotine (Ginzkey et al., 2013; Argentin and Cicchetti, 2004), EVPs (Misra et al., 2014; Takahashi et al., 2018; Tang et al., 2019) and heated tobacco (Crooks et al., 2018), but as this was an observation outside of the criteria for a positive genotoxic response, it is concluded that, under the conditions of test, both e-liquids and EVP aerosols tested in this study demonstrated no effect on MN induction. Indeed, further investigation of the genotoxic mechanisms of nicotine, reveals that these mechanisms only materialise at concentrations outside of physiologically relevant levels (Smart et al., 2019). In a recent report (Barrington-Trimis et al., 2014) the need for research on flavour additives used in EVPs was outlined, as the extent to which this product feature impacts cellular responses is not well defined. In agreement with Misra et al., 2014 and Thorne et al., 2016, the findings of the current study, for both e-liquids and EVP aerosols, did not demonstrate any effect of flavourings on micronucleus induction.

Guidelines recommend a total of at least five bacterial strains for chemical assessment (OECD guideline 471), four strains of S. typhimurium (TA98, TA100, TA1537 and TA1535) and strain TA102. The introduction of the plasmid pKM101 into the tester strains (TA98, TA100 and TA102) increases the sensitivity to certain mutagens as the plasmid codes for an error-prone DNA repair system (Maron et al., 1983, Walker, 1985). TA98 is sensitive to basic and neutral fractions, such as the heterocyclic and aromatic amines that are one of the primary sources of mutagenicity in TPM and smoke extracts. TA100 was also used because of its added sensitivities compared to TA98 and its ability to distinguish between tobacco products (Wan et al., 2009; Zeiger, 1987). All five strains were used in the current study in the assessment of e-liquids. However, as no test guideline is available for the testing of EVP aerosols, TA100 was chosen as it is the most responsive strain to EVP aerosol exposure. The use of additional strains such as TA104, in support of a more extensive testing strategy may be of value (Maron et al., 1985; Dillon et al., 1998) in the future. TA104 is sensitive to carbonyl compounds which may be of use as carbonyls can be formed during dry wicking (Farsalinos et al., 2015). As expected, all controls initiated a positive response and 3R4F smoke induced a dose dependent and significant increase in the number of revertants. No effect of e-liquid or EVP aerosol exposure was found under the conditions of the test and agrees with previous studies (Thorne et al., 2016), although, in this study, mutagenicity was assessed following bubbling of aerosol into PBS containing the tester strain rather than directly onto the surface of the cells.

Conventional tobacco smoking is known to cause a variety of diseases and further work is required to investigate the role of these assay products.
Table 6
The effect of e-liquid and EVP aerosol exposure on the fold-increase in revertant numbers with dose.

<table>
<thead>
<tr>
<th>Product</th>
<th>% Nicotine</th>
<th>TA98</th>
<th>TA100</th>
<th>TA102</th>
<th>TA1535</th>
<th>TA1537</th>
</tr>
</thead>
<tbody>
<tr>
<td>e-liquid Vapour</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blu PLUS+™</td>
<td>Base liquid</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blu GO™</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Base liquid</td>
<td>Classic tobacco</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Magnificent menthol</td>
<td>Gold leaf</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Classic tobacco</td>
<td>Wild vanilla high</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cherry crush</td>
<td>Carolina Bold</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mint chocolate</td>
<td>Carolina Bold</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vivid vanilla high</td>
<td>Carolina Bold</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Cherry crush</td>
<td>Carolina Bold</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Caramel café</td>
<td>Carolina Bold</td>
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<td>&lt;0.001</td>
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</tr>
<tr>
<td>Blueberry high</td>
<td>Carolina Bold</td>
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<td>&lt;0.001</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glacier mint</td>
<td>Carolina Bold</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Strawberry mint</td>
<td>Carolina Bold</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Slope significance was determined using a non-threshold linear regression model. *p < .05 for a significant slope. +p < .05 for one and ++ two doses compared to the zero (vehicle) control.
endpoints in disease development and the association with the use of EVPs. Studies to date demonstrate that EVPs are currently not known to be involved in the development of the same tobacco related diseases. In the current study, in which a significant reduction in cytotoxicity was observed, ranging from 143 to 1191 times less toxic, when compared to conventional tobacco smoke exposure, reduced acute toxicity may or may not reflect the toxicological effects of these products following chronic use. Longer term repeated in vitro exposure studies and, more recently, human epidemiological studies are required to fully ascertaining the safety of these products to consumers. However, the potential for these types of products to be used as aids in the cessation of tobacco smoking is encouraging and may help ameliorate or avert some of the otherwise unavoidable burdens or respiratory morbidity and mortality caused by conventional tobacco smoking.

5. Conclusion

In conclusion, tobacco smoke induced a significant and substantial increase in cytotoxicity, mutagenicity and genotoxicity in all the cells tested. For EVP aerosols, there was reduced cytotoxicity compared to cigarette smoke exposure, but no mutagenic or genotoxic response was observed when either EVP aerosols or e-liquids were tested. The data presented adds to the increasing body of scientific weight-of-evidence supporting the potential role of these high-quality EVPs as harm reduction tools for adult smokers.

Funding

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Declaration of Competing Interest

All authors are employees of Imperial Brands plc.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2020.104866.

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