Toxicology in Vitro 66 (2020) 104866

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

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

R. Wieczorek[a], G. Phillips[b,*], L. Czekala[b], E. Trelles Sticken[a], G. O'Connell[b], L. Simms[b], K. Rudd[b], M. Stevenson[b], T. Walele[b]

[a] Reemtsma Cigarettenfabriken GmbH – Imperial Brands, Albert-Einstein-Ring 7, 22761 Hamburg, Germany
[b] Imperial Brands PLC, 121 Winterstoke Road, Bristol BS3 2LL, UK

ARTICLE INFO

Keywords:
Electronic vapour product
Electronic cigarette
Tobacco
Cigarette
In vitro toxicity
E-liquids
Cytotoxicity
Genotoxicity
Mutagenicity

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-1 to 8).

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

* Corresponding author.
E-mail address: lifescitech7@gmail.com (G. Phillips).

https://doi.org/10.1016/j.tiv.2020.104866
Received 24 September 2019; Received in revised form 10 April 2020; Accepted 14 April 2020
Available online 27 April 2020

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 would be expected to elicit markedly reduced biological responses in conventional regulatory *in vitro* toxicology 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 blu 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 assay, 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 1
Test products, nicotine concentrations and exposure matrix for blu PLUS+™ and blu GO™ electronic vapour products.

Test product	Nicotine content (mg/mL)	Exposure matrix	
		Aerosol (generated with)	e-liquid
Base liquid	1.2	blu GO™, blu PLUS+™	√
Base liquid	2.4	blu GO™, blu PLUS+™	√
Classic tobacco	1.2	blu PLUS+™	√
Classic tobacco	2.4	blu GO™, blu PLUS+™	√
Gold leaf.	2.4	blu PLUS+™	√
Magnificent menthol	1.2	blu PLUS+™	√
Magnificent menthol	2.4	blu GO™, blu PLUS+™	√
Mint chocolate	2.4	blu PLUS+™	√
Vivid vanilla	2.4	blu GO™, blu PLUS+™	√
Cherry crush	1.2	blu PLUS+™	√
Cherry crush	2.4	blu GO™, blu PLUS+™	√
Caramel cafe	2.4	blu PLUS+™	√
Strawberry mint	2.4	blu PLUS+™	√
Berry cobbler	2.4	blu PLUS+™	√
Blueberry high	2.4	blu PLUS+™	√
Glacier mint	2.4	blu GO™, blu PLUS+™	√
Carolina bold	2.4	ND	√
Carolina bold	2.0	blu GO™, blu PLUS+™	√

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.

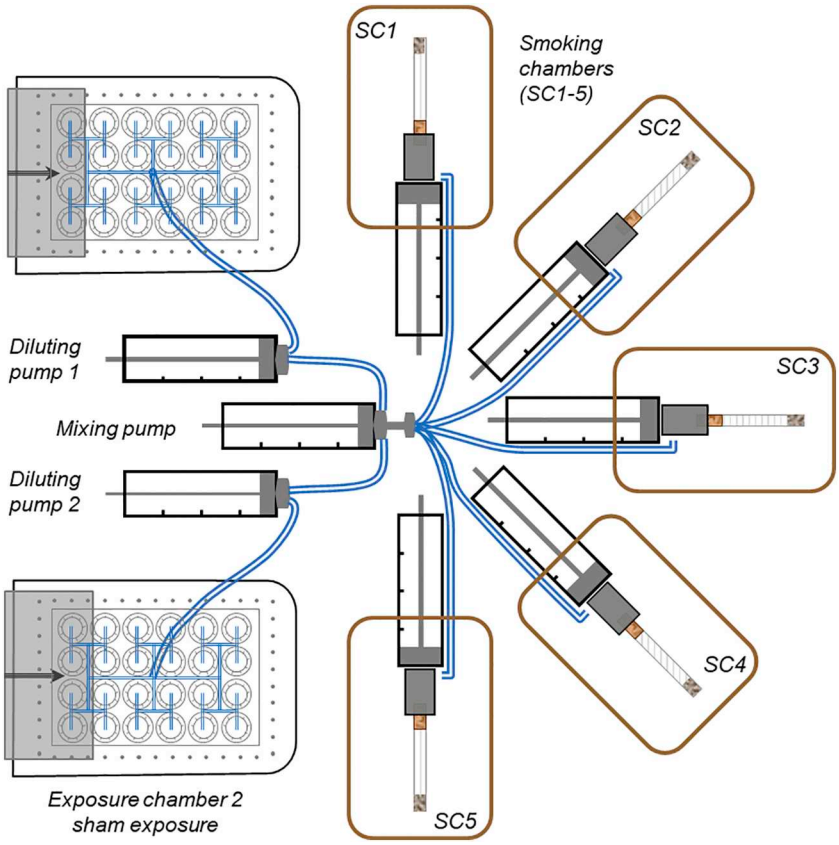

Fig. 1. *The Smoke/Aerosol Exposure In vitro System (SAEIVS) smoking machine.* The SAIEVS is an automated and computer-controlled puffing machine shown with 5 smoking chambers linked to a computer-controlled dilution system. Undiluted aerosol generated from a single syringe (within each box) is delivered into 1 mixing chamber prior to dilution in 1 or 2 diluting pumps. All dilutions are conducted at 37 °C. Following dilution tobacco smoke or EVP aerosol is delivered to 1 or 2 exposure chambers. Each chamber is connected to exhaust lines for removal of residual smoke or aerosol and prior to additional exposures.

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 L-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% CO_2.

For e-liquid exposures, 100 µL of Hep-G2 (2×10^4/mL) or Beas-2b (0.8×10^4/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% CO_2 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 CO_2 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% CO_2 for 18–24 h. Following liquid removal, 100 µL of Beas-2b (0.5×10^4/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% CO_2 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 SAIEVS 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% CO_2 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%CO_2 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_{20}) 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 (1×10^6 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_2. One millilitre of a two-fold 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_2 for 42 ± 4 h.

For the short-term treatment with and without S9, 0.5 mL of TK6 (1.5×10^6 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_2. 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_2 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 Dulbeccos'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 1×10^5 V79 cells per mL. The plates were incubated at 37 °C, in humidified 95% air, 5% CO_2 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_2 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 cytospin 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 (Vectashield 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

Table 2
In vitro micronucleus positive controls.

Positive controls	End concentration/dilution
E-Liquid exposure of TK6 cells	
Cyclophosphamide A (CPA) (Short term (ST + S9))	3. 0 µg/mL
Bleomycin (Short term (ST-S9)	0.15 µg/mL
Bleomycin (Long term (LT-S9)	0.15 µg/mL
EVP aerosol and tobacco smoke exposure of V79 cells	
Fresh whole smoke	1:4 and 1:5 (dilution)
Cyclophosphamide A (CPA)	10 µg/mL

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 micronucleus 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 micronucleus 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 mg/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 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 Ca^{2+} and Mg^{2+} free Dulbecco's PBS. A 10 mL suspension of TA100 bacterial suspension in PBS was added to an impinger 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.

Strain	Mutation	Antibiotic resistance	Positive controls	
			Without S9 (μg/plate)	With S9 (μg/plate)
TA98[a]	His D3052 (frameshift)	Ampicillin	2-Nitrofluorene (4 μg /plate)	2-Aminoanthracene (2 μg /plate)
TA100[b]	His G46 (Base pair substitution)	Ampicillin	NaAzide (1 μg /plate)	2-Aminoanthracene (2 μg /plate)
TA102[c]	His G428 (Base pair substitution)	Ampicillin, Tetracycline	Mitomycin C (1 μg /plate)	2-Aminoanthracene (8 μg /plate)
TA1535[a]	His H46 (Base pair substitution)	None	NaAzide (1 μg /plate)	2-Aminoanthracene (2 μg /plate)
TA1537[a]	His C3076 (Frameshift)	None	9-Aminoacridine (50 μg /plate)	2-Aminoanthracene (4 μg /plate)

Abbreviations: His = Histidine.
[a] National Collection of Type Cultures, Porton Down, Salisbury, UK.
[b] Covance Laboratories Inc., USA.
[c] Professor Ames' laboratory.

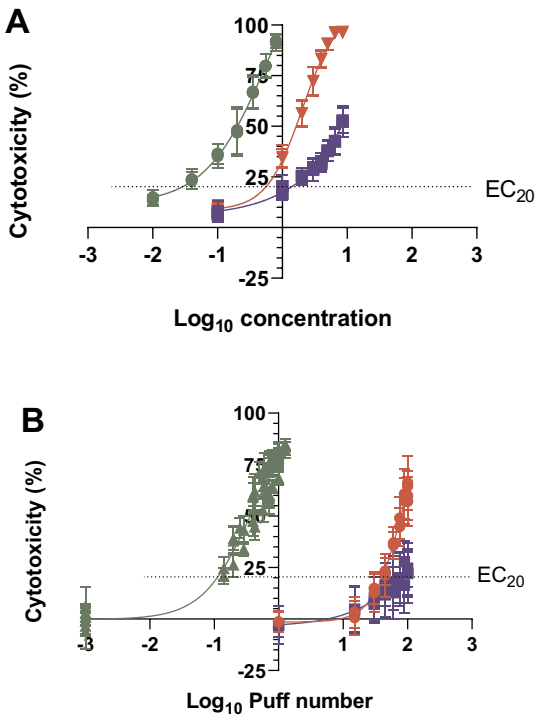

Fig. 2. *The EC_{20}determination of e-liquids, nicotine, EVP aerosols and 3R4F smoke using Beas-2b cells.* For the determination of e liquid EC_{20} (A), green dots represent nicotine, red dots Magnificent Menthol 2.4% and blue dots Base liquid 2.4%. Determination of the EC_{20} for EVP aerosols and 3R4F smoke (B), green dots represent 3R4F smoke, red dots Magnificent Menthol 2.4% aerosol and blue dots Base liquid 2.4% aerosol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 an 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 an 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 in Hep-G2 cells was significantly ($p < .05$) higher and thus less cytotoxic than the concentration from all the flavoured e-liquid products containing 1.2% nicotine (3/3) and 11 of 12 products containing 2.4% nicotine. For Beas-2b cells, only 33% (1/3) of the 1.2% nicotine and 75% (8/12) of the 2.4% nicotine flavoured e-liquid products had EC_{20} values that were significantly lower than their equivalent base liquid control (Table 4). With one exception (Mint Chocolate 2.4% nicotine), no significant effect of flavour on cytotoxicity was observed when EVP aerosols generated from the blu PLUS+™ device was assessed. Although the EC_{20} for Glacier Mint 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

Table 4
Beas-2b and Hep-G2 cytotoxicity (EC_{20}) values for e-liquids, EVP aerosol, controls and tobacco smoke.

Test material	Nicotine (%)	EC_{20}[mg/mL]	EC_{20}[mg/mL]	EC_{20}[puffs]	EC_{20}[puffs]
		Hep-G2		Beas-2b	
		e-liquids		Aerosol	
		blu PLUS + ™	blu PLUS + ™	blu PLUS + ™	blu Go™
3R4F				0.12 ± 0.02[d]	
Nicotine	100	0.05 ± 0.01[b]	0.035 ± 0.015[a]		
SDS	0.0	0.002 ± 0.0003[c]	0.003 ± 0.0003[c]		
Base liquid	1.2	2.30 ± 0.45	1.73 ± 0.62	71.83 ± 20.99	67.83 ± 17.52
Base liquid	2.4	1.44 ± 0.07***	1.54 ± 0.37	59.49 ± 5.58	54.98 ± 7.44
Classic tobacco	1.2	0.56 ± 0.34****	2.24 ± 0.76	90.51 ± 39.75	
Classic tobacco	2.4	0.39 ± 0.13****	1.18 ± 0.11	58.99 ± 14.42	30.25 ± 4.59***
Gold leaf	2.4	0.59 ± 0.16***	0.49 ± 0.14**	93.47 ± 13.19	
Magnificent menthol	1.2	0.40 ± 0.12****	0.82 ± 0.22*	100.73 ± 16.34	
Magnificent menthol	2.4	0.38 ± 0.21****	0.57 ± 0.13**	87.43 ± 15.34	39.88 ± 8.68*
Mint chocolate	2.4	0.92 ± 0.14****	0.77 ± 0.29*	143.73 ± 79.86**	
Vivid vanilla high	2.4	0.36 ± 0.05****	0.98 ± 0.25	51.89 ± 22.03	16.57 ± 3.19****
Cherry crush	1.2	0.72 ± 0.39****	1.76 ± 0.13	103.00 ± 16.86	
Cherry crush	2.4	0.64 ± 0.12**	1.07 ± 0.23	51.21 ± 36.19	45.71 ± 8.68
Caramel café	2.4	0.23 ± 0.06****	0.94 ± 0.04*	43.23 ± 16.87	
Carolina bold	2.4	1.20 ± 0.11	1.00 ± 0.07	ND	
Berry cobbler	2.4	0.18 ± 0.06****	0.70 ± 0.08***	48.49 ± 20.62	
Blueberry high	2.4	0.37 ± 0.11****	0.84 ± 0.26**	51.08 ± 32.55	
Glacier mint	2.4	0.50 ± 0.10***	0.81 ± 0.13**	95.05 ± 22.10	76.37 ± 8.68**
Strawberry mint	2.4	0.79 ± 0.20*	0.73 ± 0.01**	48.88 ± 14.21	

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

[a] $p < .05$ 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 Cobbler 2.4%, Blueberry 2.4% and Glacier Mint 2.4%.

[b] $p < .05$ for all blu PLUS+™ e-liquid products assessed in Hep-G2 cells except for Gold leaf 2.4% and Magnificent Menthol 2.4%.

[c] $p < .05$ for all e-liquid products.

[d] $p < .05$ compared to all devices generating aerosol.

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 (TA1537), S9 treatment resulted in higher revertant numbers than without S9. For 3R4F smoke exposure there was a significant dose (puff) dependent in increase revertant numbers (Fig. 8B).

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 *S. typhimurium* strains. Of the eight identified significant slopes, three were negative and of the remaining five only Caramel café (2.4% nicotine) e-liquid induced a corresponding increase in revertant numbers at two concentrations. However, this was only found in the TA102 strain and was not above the two-fold increase required under the acceptance criteria. As none of the acceptance criteria were met for either the e-liquid or EVP aerosol response, all products were considered non-mutagenic regardless of exposure matrix.

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.

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 EC_{20} as it was not possible to derive the EC_{50} (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 EC_{20} 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

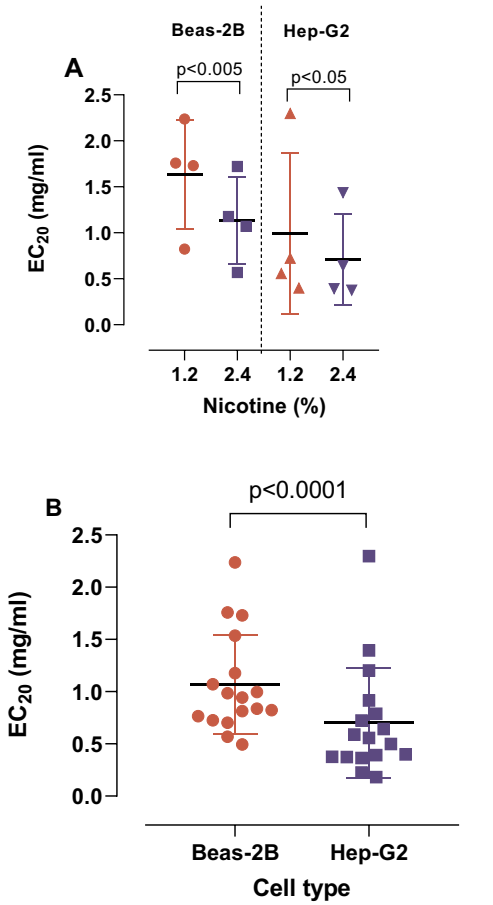

Fig. 3. *The effect of nicotine (A) and cell type (B) on the cytotoxicity (EC_{20}) of Beas-2b and Hep-G2 cells following e-liquid exposure.* There was a significant ($p < .005$ and $p < .05$) effect of nicotine (A) and cell type ($p < .0001$) on cytotoxicity following e-liquid exposure.

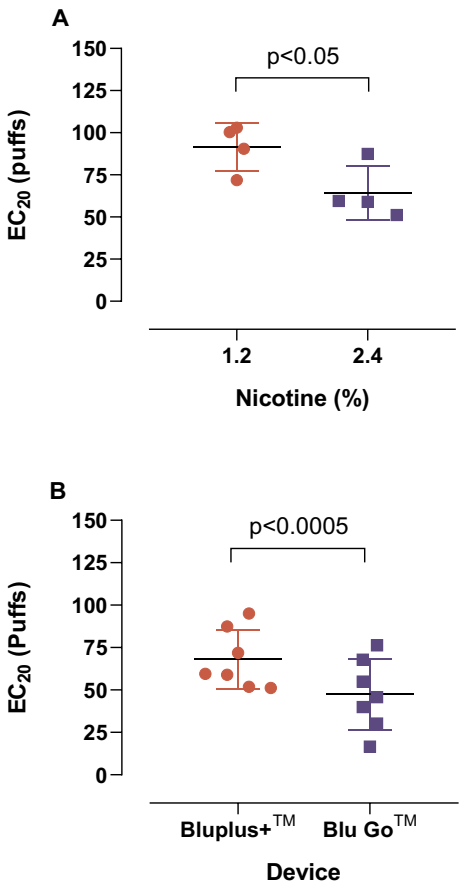

Fig. 4. *The effect of percent nicotine and device on the cytotoxicity of EVP aerosol exposure of Beas-2b cells.* Equivalent e-liquids (Base Liquid, Magnificent Menthol, Classic Tobacco and Cherry Crush) containing 1.2% and 2.4% nicotine and EVP aerosol devices were compared. There was a significant effect of nicotine (A: $p < .05$) and device (B: $p < .0005$) on cytotoxicity following exposure to EVP 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 known have profound effects on a cell's response to exposure (Briske-Anderson et al., 1987 and Azzopardi et al., 2015). Further studies are required, including the use of submerged incubator controls, to demonstrate the robustness of the cellular systems used and to assess the 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

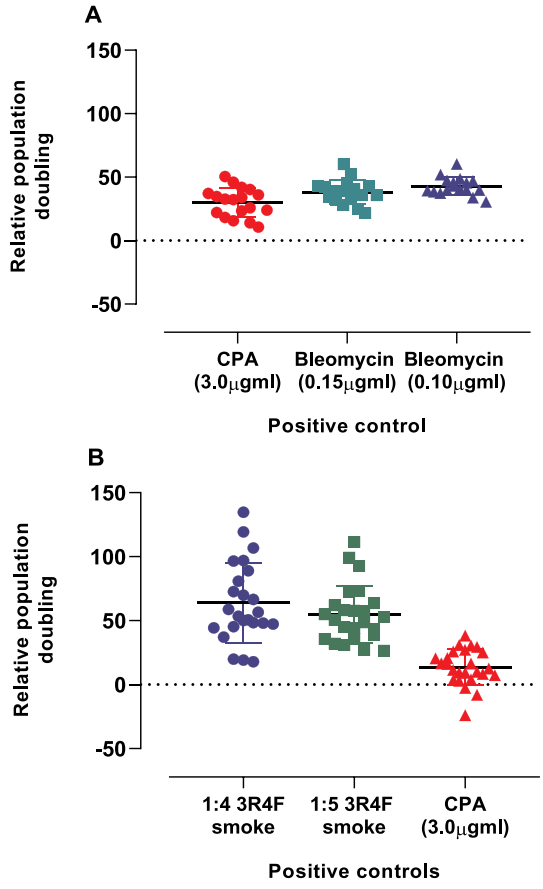

Fig. 5. *The effect of e-liquid (A) and EVP aerosol (B) positive controls on cytotoxicity and IVM.* Abbreviations; CPA - Cyclophosphamide A.

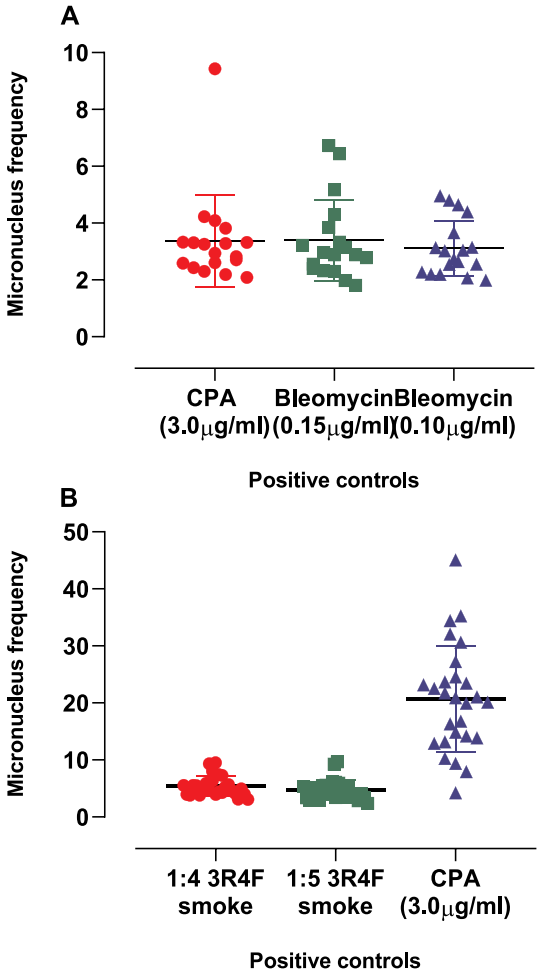

Fig. 6. *The effect of e-liquid (A) and EVP aerosol (B) positive controls on micronucleus frequency.* Micronucleus frequency based on fold change in revertants relative to vehicle control. Abbreviations. CPA – Cyclophosphamide A.

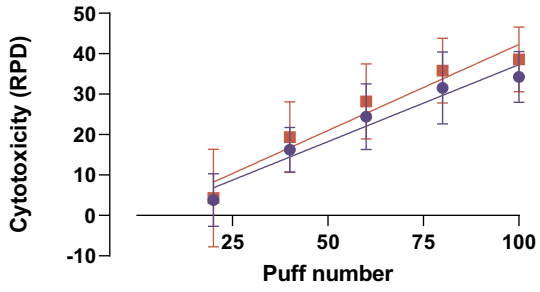

Fig. 7. *The effect of EVP aerosol exposure generated from blu PLUS + ™ and blu GO™ on cytotoxicity.* Red squares relate to blu GO™ and blue circles to blu PLUS +™. There was a significant ($p < .0001$) effect of puff number on cytotoxicity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 todate, 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).

Table 5
The effect of e-liquid and EVP aerosol exposure on *in vitro* micronucleus frequency dose response.

Product	*E*-Liquids			Aerosol	
	BluPlus + ™ST + S9	BluPlus + ™ST-S9	BluPlus + ™LT-S9	BluPlus + ™ST + S9	Blu Go™ST + S9
	Slope	Slope	Slope	Slope	Slope
Base liquid 1.2%	0.059	0.039	0.007	0.001	−0.001
Base liquid 2.4%	0.181*	0.123**++	0.012	0.001	−0.001
Cherry crush 1.2%	0.004	−0.023	−0.037	−0.001	
Cherry crush 2.4%	−0.011	−0.095**+	−0.01	0.001	−0.001
Magnificent menthol 1.2%	−0.036++	−0.097+	−0.020	−0.001	
Magnificent menthol 2.4%	−0.084	−0.154	−0.067	0.000	0.002
Classic tobacco 1.2%	0.0222	0.030	0.103+	0.001	
Classic tobacco 2.4%	0.055	−0.085++++	−0.051	−0.001	0.002
Caramel café 2.4%	−0.023	0.194	0.039	0.001	
Carolina bold 2.4%	−0.060	0.020	−0.028	ND	0.001
Gold leaf 2.4%	−0.016	0.353	0.069	−0.001	
Berry cobbler 2.4%	−0.073	0.152**	0.127+	0.001	
Blue berry 2.4%	0.062	0.014	0.073	0.001	
Glacial mint 2.4%	0.277	0.149	0.105*	−0.001	−0.001
Mint chocolate 2.4%	−0.085*+	0.266	−0.057	−0.001	
Strawberry mint 2.4%	0.007	0.002	−0.022	0.001	0.001
Vivid vanilla 2.4%	0.058	−0.055	−0.050+	−0.001	0.001

Slopes were calculated on the micronuclei frequency-dose response curves. *$p < .05$, **$p < .005$; effect of slope and $^{+}p < .05$ effect of dose (+ = 1 dose, + + = 2 doses, + + + + = 4 doses) compared to vehicle control.

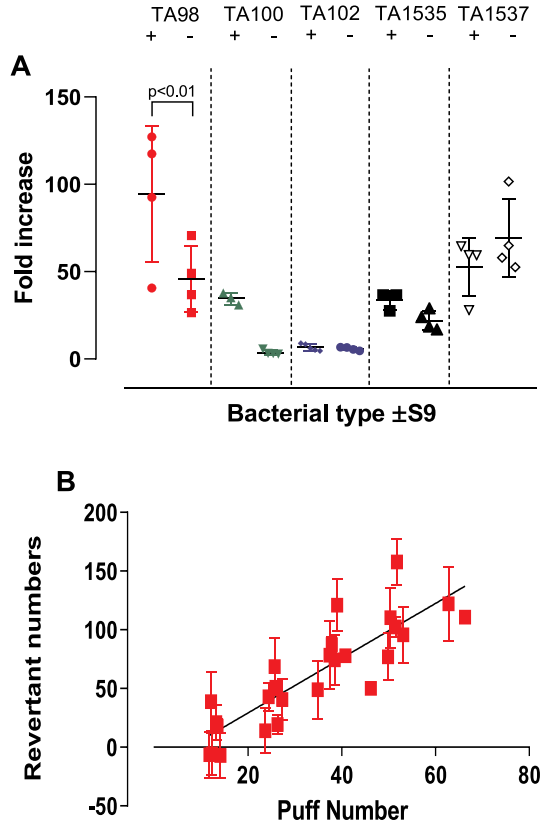

Fig. 8. *The effect of exposure to positive controls on revertant numbers and with and without S9 in five Salmonella typhimurium strains used for e-liquid (A) and 3R4F smoke (B).* There was a seven-fold (TA102) to ninety-four-fold (TA98) increase in revertant numbers following +S9 treatment, which was significant ($p < .01$) for TA98 only. There was a significant ($p < .0001$) increase in revertant numbers with increasing puff number using TA100.

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, TA1535 and TA1537) 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 (Marnett 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

Table 6
The effect of e-liquid and EVP aerosol exposure on the fold-increase in revertant numbers with dose.

Product	% Nicotine	TA98		TA100				TA102		TA1535		TA1537	
		e-liquid		Vapour		e-liquid		e-liquid		e-liquid		e-liquid	
				Blu PLUS+™	Blu GO™								
		+S9	-S9	+S9	+S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Base liquid	1.2	< −0.001	< −0.001^{+}	< 0.001	< 0.001	−0.001	−0.003	0.004	0.005	< 0.001	< 0.001	< −0.001	0.0
Base liquid	2.4	0.002	< −0.001	< 0.001	< 0.001	0.001	< −0.001	0.01	0.003	0.0	0.001	< 0.001	< 0.001
Classic tobacco	1.2	< 0.001	< −0.001	< 0.001		−0.001	0.004	0.009	−0.005	0.002*	< 0.001	0.0	< 0.001
Classic tobacco	2.4	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	−0.002	−0.001	0.004	< 0.001	< 0.001	< −0.001	< 0.001
Gold leaf	2.4	0.001	< −0.001^{+}	< 0.001		−0.004	−0.003	−0.015*	−0.01^{+}	0.001	0.002	< −0.001	< −0.001
Magnificent menthol	1.2	0.002	0.0	< 0.001*		0.001	−0.006	−0.005^{+}	0.009	< 0.001	< −0.001	< 0.001	0.001
Magnificent menthol	2.4	0.001	0.001	< 0.001	< 0.001	−0.002	−0.001	0.026	−0.006	0.001	< 0.001	0.001^{++}	< 0.001
Mint chocolate	2.4	< −0.001	< 0.001	< 0.001		0.003	−0.004	−0.008	0.003	< 0.001*	< 0.001	< 0.001	< 0.001
Vivid vanilla high	2.4	−0.002	< −0.001	< 0.001	< 0.001	0.002	0.001	−0.006	−0.001	< 0.001	< 0.001	< 0.001	< 0.001
Cherry crush	1.2	−0.002	0.002	< 0.001		−0.002	−0.001	< −0.001	< 0.001	0.001	< 0.001	< −0.001	< 0.001
Cherry crush	2.4	< −0.001^{+}	< −0.001	< 0.001	< 0.001	−0.001	0.002	−0.006^{+}	0.009	< 0.001	−0.001	−0.002*	< −0.001
Caramel café	2.4	−0.002	< 0.001	< 0.001		−0.002	−0.004*	0.015^{*++}	−0.006	< 0.001^{+}	< −0.001	< −0.001	< −0.001
Carolina Bold	2.4	0.002	−0.001	< 0.001		0.002	0.003	−0.002	< −0.001	< −0.001	0.0	< 0.001	< 0.001
Berry cobbler	2.4	< −0.001	0.0	< 0.001*		< −0.001	−0.001	−0.006	−0.002	< 0.001	−0.001	0.001	< 0.001
Blueberry high	2.4	< 0.001	0.002	< 0.001		0.002	−0.001	0.017*	−0.004	< −0.001^{+}	−0.001	< 0.001	< −0.001
Glacier mint	2.4	< −0.001	0.0	< 0.001	< 0.001	< 0.001	−0.003^{+}	−0.003	< 0.001	< −0.001	< 0.001	< −0.001	< −0.001
Strawberry mint	2.4	−0.001	< 0.001	< 0.001		−0.003	< 0.001	−0.01^{++}	−0.005	0.001^{+}	< −0.001	< 0.001	< 0.001
Carolina bold	2.0	0.001	< −0.001		< 0.001	−0.002	0.004*	0.004^{+}	−0.002^{+}	< −0.001	< −0.001	−0.002	−0.001

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 risk associated 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 relevantly, human epidemiological studies are required to fully ascertain 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 are 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 nor 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

This study was funded by Imperial Brands plc. the manufacturer of the EVPs used in this study, is a wholly owned subsidiary of Imperial Brands plc.

Declaration of Competing Interest

All authors are employees of Imperial Brands plc.

Acknowledgments

We thank the analytical testing laboratories at Imperial Brands plc for performing the neat e-liquid, smoke and aerosol testing.

Appendix A. Supplementary data

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

References

Argentin, G., Cicchetti, R., 2004. Genotoxic and antiapoptotic effect of nicotine on human gingival fibroblasts. Toxicol. Sci. 79, 75–81.
Azzopardi, D., Haswell, L., Foss-Smith, G., Hewitt, K., Asquith, N., Corke, S., Phillips, G., 2015. Evaluation of an air–liquid interface cell culture model for studies on the inflammatory and cytotoxic responses to tobacco smoke aerosols. Toxicol. in vitro 29, 1720–1728.
Bahl, V., Linb, S., Xub, N., Davis, B., Wang, Y., Talbota, P., 2012. Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models. Reprod. Toxicol. 34, 529–537.
Barrington-Trimis, J., Samet, J., McConnell, R., 2014. Flavorings in electronic cigarettes: an unrecognized respiratory health hazard? JAMA 17 (312(23)), 2493–2494.
Biener, L., Hargraves, L., 2014. A longitudinal study of electronic cigarette use among a population-based sample of adult smokers: Association with smoking cessation and motivation to quit. Nicot. Tobac. Res. 127–133.
Bonassi, S., El-Zein, R., Bolognesi, C., Fenech, M., 2011 Jan. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. Mutagenesis 26 (1), 93–100.
Borenfreund, E., Puerner, J.A., 1985. Toxicity determined *In vitro* by morphological alterations and neutral red absorption. Toxicol. Lett. 24 (2–3), 119–124.
Briske-Anderson, M., Finley, J., Newman, S.M., 1997. The influence of culture time and passage number on the morphological and physiological development of Caco-2 cells. Proc. Soc. Exp. Biol. Med. 214 (3), 248–257.
Chen, C., Lee, S., Yu, H., Huang, F., Chang, Y., 2015. Effects of nicotine on cell growth, migration, and production of inflammatory cytokines and reactive oxygen species by cementoblasts. J. Dental Sci. 10 (2), 154–160.
COM, 2011. Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment. In: Guidance on a strategy for genotoxicity testing of chemical substances, . http://www.iacom.org.uk/guidstate/documents/COM.GuidanceFINAL2.pdf.
Combes, R., Scott, I., Crooks, I., Dillon, D., Meredith, C., McAdam, K., Proctor, C., 2013. The *in vitro* cytotoxicity and genotoxicity of cigarette smoke particulate matter with reduced toxicant yields. Toxicol. *in vitro* 27, 1533–1541.
CORESTA, 2004. *In vitro* Toxicology Task Force: the Rationale and Strategy for Conducting *in vitro* Toxicology Testing of Tobacco Smoke. https://www.coresta.org/rationale-and-strategy-conducting-vitro-toxicology-testing-tobacco-smoke-29237.html.
CORESTA, 2015. Recommended Method No. 81. Routine Analytical Machine for *E*-Cigarette Aerosol Generation and Collection - Definitions and Standard Condition. Available from. http://www.coresta.org/Recommended_Methods/CRM_81.pdf.
Crooks, I., Dillon, D., Scott, J., Ballantyne, A., Meredith, C., 2013. The effect of long term storage on tobacco smoke particulate matter in *in vitro* genotoxicity and cytotoxicity assays. Regul. Toxicol. Pharmacol. 65, 196–200.
Crooks, I., Neilson, L., Scott, K., Reynolds, L., Oke, T., Forster, M., Meredith, C., McAdam, K., Proctor, C., 2018. Evaluation of flavourings potentially used in a heated tobacco product: Chemical analysis, *in vitro* mutagenicity, genotoxicity, cytotoxicity and *in vitro* tumour promoting activity. Food Chem. Toxicol. 118, 940–952.
Czekala, L., Simms, L., Stevenson, M., Trelles-Sticken, E., Walker, P., Walele, T., 2019. High content screening in NHBE cells shows significantly reduced biological activity of flavoured e-liquids, when compared to cigarette smoke condensate. Toxicology *in vitro* 58, 86–96.
Dillon, D., Combes, R., Zeiger, E., 1998. The effectiveness of salmonella strains TA100: TA102 and TA104 for detecting mutagenicity of some aldehydes and peroxides. Mutagenesis 13, 19–26.
Farsalinos, K., Romagna, G., Tsiapras, D., Kyrzopoulos, S., Spyrou, A., Voudris, V., 2013. Impact of flavour variability on electronic cigarette use experience: An internet survey. Int. J. Environ. Res. Public Health 10, 7272–7282.
Farsalinos, K., Voudris, V., Poulas, K., 2015. *E*-cigarettes generate high levels of aldehydes only in 'dry puff' conditions. Addiction 110, 1352–1356.
Fenech, M., 1993. The cytokinesis-block micronucleus technique: a detailed description of the method and its application to genotoxicity studies in human populations. Mutat. Res. 285 (1), 35–44.
Fowler, P., Smith, R., Smith, K., Young, J., Jeffrey, L., Kirkland, D., Pfuhlerd, S., Carmicheal, P., 2012. Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays. II. Importance of accurate toxicity measurement. Mutat. Res. 747 (1), 104–117.
Fowler, P., Smith, R., Smith, K., Young, J., Jeffrey, L., Carmichael, P., Kirkland, K., Pfuhler, S., 2014. Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays. III: Sensitivity of human cell types to known genotoxic agents. Mutat. Res. 767, 28–36.
Ginzkey, C., Friehs, G., Koehler, C., Hackenberg, S., Hagen, R., Kleinsasser, N., 2013. Assessment of nicotine-induced DNA damage in a genotoxicological test battery. Mutat. Res. 751, 34–39.
Goniewicz, M., Knysak, J., Gawron, M., Kosmider, L., Sobczak, A., Kurek, J., Prokopowicz, A., Jablonska-Czapla, M., Rosik-Dulewska, C., Havel, C., Jacob, P., Benowitz, N., 2014. Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. Tob. Control 23 (2), 133–139 Mar.
Gonzalez-Suarez, I., Marescotti, D., Martin, F., Scotti, E., Guedj, E., Acali, S., Dulize, R., Baumer, K., Peric, P., Frentzel, S., Ivanov, N., Hoeng, J., Peitsch, M., 2017. *In vitro* systems toxicology assessment of nonflavored e-cigarette liquids in primary lung epithelial cells. Appl. *In vitro* Toxicol. 3 (1), 41–55.
https-1 www8.nationalacademies.org/onpinews/newsitem.aspx?RecordID=24952.
https-2 www.fda.gov/newsevents/newsroom/pressannouncements/ucm605432.htm.
https-3 www.cancer.org/healthy/stay-away-from-tobacco/e-cigarette-position-statement.html.
https-4 www.canada.ca/en/health-canada/services/smoking-tobacco/vaping.html.
https-5 www.bma.org.uk/collective-voice/policy-and-research/public-and-population-health/tobacco/e-cigarettes.
https-6 www.health.govt.nz/our-work/preventative-health-wellness/tobacco-control/vaping-smokeless-including-heated-tobacco.
https-7 www.ranzcp.org/Files/Resources/Submissions/RANZCP_Standing-Committee_Vaporised-Nicotine-Bill.aspx.
https-8 www.danaonline.org/wp-content/uploads/2017/09/DANA-Position-Statement-on-E-Cigarettes-2017.pdf.
ICH, 2011. ICH Harmonised Tripartite Guideline: Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use S2(R1). http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S2_R1/Step4/S2R1_Step4.pdf.
International Organisation for Standerdisation, 1999. ISO 3402:1999. Tobacco and tobacco products - Atmosphere for conditioning and testing. https://www.iso.org/standard/28324.html.
Kado, N., Langley, D., Eisenstadt, E., 1983. A simple modification of the Salmonella liquid-incubation assay. Increased sensitivity for detecting mutagens in human urine. Mutat. Res. Jul;121 (1), 25–32.
Le Godec, T., Crooks, I., Scott, K., Meredith, C., 2019. *In vitro* mutagenicity of gas-vapour phase extracts from flavoured and unflavoured heated tobacco products. Toxicol. Rep. 6, 1155–1163.
Leigh, N., Lawton, R., Hershberger, P., Goniewicz, M., 2016. Flavourings significantly affect inhalation toxicity of aerosol generated from electronic nicotine delivery systems (ENDS). Tob. Control 25 (2), 81–87.

Li, X., 2016. *In vitro* toxicity testing of cigarettete smoke based on the air-liquid interface exposure: A review. Toxicol. *in vitro* 36, 105–113.
Marnett, L., Hurd, H., Hollstein, M., Levin, D., Esterbauer, H., Ames, B., 1985. Naturally occuring carbonyl compounds are mutagens in salmonella tester strain 104. Mutat. Res. 148, 25–34.
Maron, D., Ames, B., 1983. Revised methods for the *Salmonella* mutagenicity test. Mutat. Res. 113, 173–215.
McNeill, A., Brose, L., Calder, R., Hitchman, S., Hajek, P., McRobbie, H., 2015. *E*-cigarettes: An Evidence Update (Public Health England 2015 report). Public Health England.
McNeill, A., Brose, L., Calder, R., Bauld, L., Robson, D., 2018. Evidence review of e-cigarettes and heated tobacco products 2018. A report commissioned by Public Health England. London.
Misra, M., Leverette, R., Cooper, B., Bennett, M., Brown, S., 2014. Comparative *in vitro* toxicity profile of electronic and tobacco cigarettes,. smokeless tobacco and nicotine replacement therapy products: E-liquids, extracts and collected aerosols. Int. J. Environ. Res. Public Health 11, 11,325–11,347.
Moga, M., Bosca, A., Soritau, O., Baciut, M., Lucaciu, O., Virag, P., Ilea, A., Dirzu, N., Septimiu, M., Campian, S., 2016. Nicotine cytotoxicity on the mesenchymal stem cells derived from human periodontium. Roman. Biotechnol. Lett 21 (4).
OECD, 1997. Test No. 471: Bacterial Reverse Mutation Test, OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. OECD Publishinghttps://doi.org/10.1787/9789264071247-en.
OECD, 2016. Test No. 487. *In vitro* Mammalian Cell Micronucleus Test, OECD Guidelines for the testing of chemicals, Section 4: Health effects. OECD Publishinghttps://doi.org/10.1787/9789264264861-en.
Omaiye, E., McWhirter, K., Luo, W., Tierney, P., Pankow, J.F., Talbort, P., 2019. High concentrations of flavor chemicals are present in electronic cigarette refill fluids. Sci. Rep. 9 (1), 2468 2019 Feb 21.
Royal College of Physicians(London) & Tobacco Advisory Group, 2016. Nicotine Without Smoke: Tobacco Harm Reduction. Available from. https://www.rcplondon.ac.uk/projects/outputs/nicotine-without-smoke-tobacco-harm-reduction-0.
Scheffler, S., Dieken, H., Krischenowski, O., Aufderheide, M., 2015. Cytotoxic evaluation of e-liquid aerosol using different lung-derived cell models. Int. J. Environ. Res. Public Health 12, 12,466–12,474.
Smart, D., Helbling, F., Verardo, M., Da, McHugh, Vanscheeuwijck, P., 2019. Mode-of-action analysis of the effects induced by nicotine in the *in vitro* micronucleus assay. Environ. Mol. Mutagen 60 (9), 778–791.
Stabile, A., Marinucci, L., Balloni, S., Giuliani, A., Pistilli, A., Bodo, M., Rende, M., 2018. Long term effects of cigarette smoke extract or nicotine on nerve growth factor and its receptors in a bronchial epithelial cell line. Toxicol. *In vitro* 53, 29–36.
Takahashi, T., Kanemaru, Y., Fukushima, T., Eguchi, K., Yoshida, S., Miller-Holt, J., Jones, I., 2018. Chemical analysis and *in vitro* toxicological evaluation of aerosol from a novel tobacco vapor product: A comparison with cigarette smoke. Regul. Toxicol. Pharmacol. 92, 94–103.
Tang, M.-S., Wu, X.-R., Lee, H.-W., Xia, Y., Deng, F.-M., Moreira, A., Chen, L.-C., Huang, W., Lepor, H., 2019. Electronic-cigarette smoke induces lung adenocarcinoma and bladder urothelial hyperplasia in mice. PNAS October 22, 116 (43), 21727–21731.
Tayyarah, R., Long, G., 2014. Comparison of select analytes in aerosol from e-cigarettes with smoke from conventional cigarettes and with ambient air. Regul. Toxicol. Pharmacol. Dec;70 (3), 704–710.
Thorne, D., Kilford, J., Hollings, M., Dalrymple, A., Ballantyne, M., Meredith, C., Dillon, D., 2015. The mutagenic assessment of mainstream cigarette smoke using the ames assay: A multi-strain approach. Mutat. Res./Genet. Toxicol. Environ. Mutagen. 782, 9–17.
Thorne, D., Crooks, L., Hollings, M., Seymour, A., Meredith, C., Gaca, M., 2016. The mutagenic assessment of an electronic cigarette and reference cigarette smoke using the Ames assay in strains TA98 and TA100. Mutat. Res 812, 29–38.
Thorne, D., Leverette, R., Breheny, D., Lloyd, M., McEnaney, S., Whitwell, J., Clements, J., Bombick, B., Gaca, M., 2019. Genotoxicity evaluation of tobacco and nicotine delivery products: Part Two. *In vitro* micronucleus assay. Food Chem. Toxicol 132, 110546.
USFDA, 2012. U.S. Food and Drug Administration, Center for Tobacco Products (CTP), Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke; under section 904 (a)(3) of the Federal Food, drug and Cosmetic Act. Available from. http://www.fda.gov/TobaccoProducts/Guidance.ComplianceRegulatoryInformation/ucm297786.htm.
Vlasceanu, A., Baconi, D., Galateanu, Miriana S., Balalau, C., 2018. Comparative cytotoxicity study of nicotine and cotinine on MRC-5 cell line. J. Mind. Med. Sci. 5 (1), 117–122.
Walker, G., 1985. Mutagenesis-enhancement by plasmids in mutagenesis tester strains. Basic Life Sci. 34, 111–120.
Wan, J., Johnson, M., Schilz, J., Djordjevic, M., Rice, J., Shields, P., 2009. Evaluation of *in vitro* assays for assessing the toxicity of cigarette smoke and smokeless tobacco. Cancer Epidemiol. Biomarkers Prev. 18 (12), 3263–3304.
Whitwell, J., Smith, R., Jenner, K., Lyon, H., Wood, D., Clements, J., Pfuhler, S., 2015. Relationships between p53 status, apoptosis and induction of micronuclei in different human and mouse cell lines *in vitro*: Implications for improving existing assays. Mutat. Res./Genet. Toxicol. Environ. Mutag. 789, 7–27.
Zeiger, E., 1987. Carcinogenicity of mutagens: Predictive capability of the salmonella mutagenesis assay for rodent carcinogenicity. Cancer Res. 47, 1287–1296.