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The *in vitro* ToxTracker and Aneugen Clastogen Evaluation extension assay as a tool in the assessment of relative genotoxic potential of e-liquids and their aerosols

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Abstract

In vitro (geno)toxicity assessment of electronic vapour products (EVPs), relative to conventional cigarette, currently uses assays, including the micronucleus and Ames tests. Whilst informative on induction of a finite endpoint and relative risk posed by test articles, such assays could benefit from mechanistic supplementation. The ToxTracker and Aneugen Clastogen Evaluation analysis can indicate the activation of reporters associated with (geno)toxicity, including DNA damage, oxidative stress, the p53-related stress response and protein damage. Here, we tested for the different effects of a selection of neat e-liquids, EVP aerosols and Kentucky reference 1R6F cigarette smoke samples in the ToxTracker assay. The assay was initially validated to assess whether a mixture of e-liquid base components, propylene glycol (PG) and vegetable glycerine (VG) had interfering effects within the system. This was achieved by spiking three positive controls into the system with neat PG/VG or phosphate-buffered saline bubbled (bPBS) PG/VG aerosol (nicotine and flavour free). PG/VG did not greatly affect responses induced by the compounds. Next, when compared to cigarette smoke samples, neat e-liquids and bPBS aerosols (tobacco flavour; 1.6% freebase nicotine, 1.6% nicotine salt or 0% nicotine) exhibited reduced and less complex responses. Tested up to a 10% concentration, EVP aerosol bPBS did not induce any ToxTracker reporters. Neat e-liquids, tested up to 1%, induced oxidative stress reporters, thought to be due to their effects on osmolarity *in vitro*. E-liquid nicotine content did not affect responses induced. Additionally, spiking nicotine alone only induced an oxidative stress response at a supraphysiological level. In conclusion, the ToxTracker assay is a quick, informative screen for genotoxic potential and mechanisms of a variety of (compositionally complex) samples, derived from cigarettes and EVPs. This assay has the potential for future application in the assessment battery for next-generation (smoking alternative) products, including EVPs.

Introduction

In recent years, a growing range of next-generation products (NGPs) have been developed to enable adult cigarette smokers' transition to potentially less harmful modes of nicotine delivery (1–3). Electronic vapour products (EVPs) are one such category of NGP and have been posited to be 95% less harmful than cigarette smoking (1,2). EVPs vaporise e-liquids to produce an aerosol inhalable by the user; the base constituents of these liquids are typically propylene glycol (PG) and vegetable glycerine (VG), with additional water, nicotine and flavourings (4,5). Prior to their potential launch, such products and their ingredients are subject to a risk assessment process. This can include literature-based evaluation of ingredients and determination of e-liquid composition, followed by (*in vitro*) biological testing of both the EVP liquids and aerosols if necessary. Genotoxicity testing, for example, with the regulatory-accepted *in vitro* micronucleus and Ames bacterial reverse mutation assays, forms an important part of this biological assessment. However, whilst these tests measure the induction of finite endpoints, they could benefit from supplementary screening for further insight into the mechanisms involved, particularly in the case of positive or equivocal results. Additional mechanistic assessment would also act as a confirmatory assessment for negative outcomes.

In line with a high-throughput NGP development approach, novel *in vitro* risk assessment tools should ideally be medium/high content, quick and sensitive and provide mechanistic resolution (6). A number of higher-throughput mechanistic assays are available for (geno)toxicity testing (7–11). In particular, the ToxTracker assay offers an advantage of using genetically stable but highly sensitive mouse embryonic stem (mES) cells (10,12). Whilst they are a valuable, and currently indispensable, tool *in vitro*, immortalised cell lines commonly used in genotoxicity assays, including human HepG2 and TK6, are often cancer derived. Therefore, they exhibit genetic instability and population heterogeneity, which may increase with passaging (10,13). The mES cells used in the ToxTracker assay have a number of advantages over these as previously described (10,12). Within the ToxTracker assay, mES cells have been modified with bacterial artificial chromosome recombineering to produce six green fluorescent protein (GFP) cellular stress reporter cell lines (9,10). The six human-relevant reporters include two DNA damage markers, Bsc12-GFP (associated with damage-causing DNA replication stress (9)) and Rtkn-GFP (indicating DNA double strand breaks) (10). Oxidative stress is indicated by Nrf2 target, Srxn1-GFP and/ or Nrf2-independent Blvr-GFP (10,14–16). Btg2-GFP, associated with the p53 response, also indicates cellular stress (10), and protein damage, associated with carcinogenesis, is indicated by unfolded protein response (UPR) associated Ddit3-GFP (10,17–19). Additional extension of the ToxTracker assay with cell cycle and aneuploidy analysis in wild-type mES cells [ToxTracker-Aneugen Clastogen Evaluation (ACE)] enables detection of changes such as those known to be associated with an aneugenic mode of action (11,20,21). ToxTracker has been extensively validated for its sensitivity (95%) and specificity (94%) with >450 carcinogenic and non-carcinogenic compounds (10,22,23). Furthermore, the assay has been used to assess mechanistically complex samples, such as nanomaterials (17,24,25), demonstrating its potential for the testing of novel test articles, including those produced from EVPs.

In vitro EVP risk assessment usually considers both the e-liquid and its aerosol, using reference cigarette smoke extracts or whole smoke as a comparator. *In vitro* exposure methods in the testing of cigarette- or NGP-derived samples are varied (4,26–30). Exposure to whole smoke/aerosol may be the most comprehensive, providing all

or most of their components to the cells. Additionally, this exposure is often achieved at the air-liquid interface using lung-derived cells, adding physiological relevance to the outcome (29,31). However, this method does not always complement a high-throughput screening approach due to limits in the number of wells of cells that can be exposed, and the variety of test articles that can be used, at any one time. Trapping of aerosol or smoke by bubbling through an aqueous medium, such as phosphate-buffered saline (PBS) solution, can offer some physiological relevance whilst providing a format for easy addition to (2D) cell cultures and can be generated in relatively high volumes. Neat e-liquids have been demonstrated to have effects *in vitro*, such as increasing solution osmolarity (26,28,32). As neat e-liquid testing also forms a part of the risk assessment process, for a novel assay to be suitable for such purposes, it must withstand their addition to the system.

Nicotine can be delivered by EVPs in either a freebase or salt form. In the form of a weak base, freebase, or unprotonated, nicotine is mainly absorbed in the mouth and upper respiratory tract (33). The more recent innovation of delivering 'nicotine salts' in the EVP aerosol allows greater nicotine delivery and absorption into the lungs of adult consumers and, therefore, higher plasma nicotine concentrations (33). Nicotine salts aim to more closely replicate combustible cigarette-like nicotine delivery in the lungs, thereby providing greater satisfaction to adult smokers considering ENDS as an alternative to combustible cigarettes (33). The nicotine lactate salt is present in the e-liquid, pre-formed by the addition of a suitable acid to nicotine. This difference (in nicotine form) constitutes a (minor) change in EVP formulation. Further to this, when tested alone, nicotine has been demonstrated to have a number of toxic effects both *in vitro* and *in vivo* (3,26,34) and, in particular, has demonstrated genotoxic effects *in vitro*, albeit at supraphysiological concentrations (3).

This study aimed to assess the effects of tobacco-flavoured EVP samples (0% nicotine, 1.6% nicotine salt or 1.6% freebase nicotine), either in the form of neat e-liquids or aerosol bPBS, in the ToxTracker assay, compared to Kentucky 1R6F reference cigarette smoke samples. The study also aimed to characterise the effects of selected components of the test articles: (i) neat PG/VG mix or PG/VG mix aerosol bPBS (alongside selected positive control compounds) and (ii) pure nicotine.

Materials and methods

Sample naming conventions

Test article preparation

Samples (Table 1) were generated from the 1R6F reference cigarette (Batch No. V062X53D1; University of Kentucky Centre for Tobacco Reference Products, USA) or from experimental e-liquids manufactured using materials from the usual supply chain by Nerudia (UK; Table 2). The 1R6F cigarettes were stored at 4°C in an airtight container and prior to use were equilibrated to room temperature for 15 min before conditioning in a standard humidified chamber for at least 48 h, according to the International Organization for Standardization (ISO) Guideline 3402 (35). myblu™ closed pod-system EVPs, consisting of two segments [a rechargeable battery section (350 mAh battery capacity) and a replaceable pod containing e-liquid (1.5 ml; 1.3 Ω coil resistance), were stored at room temperature until, and fully charged before, use.

Cigarette (1R6F) smoke and EVP aerosol were generated using a Vitrocell VC 10 S-Type smoking machine (Vitrocell, Germany). The smoke or aerosol was extracted by bubbling through three in-line

Table 1. Details of the test samples used in this study, derived from either EVPs or the 1R6F reference cigarette

| Test article | Description |
|----------------|---|
| EVP-neat-NS-TF | Neat e-liquid base containing 1.6% nicotine salt and tobacco flavouring |
| EVP-neat-FB-TF | Neat e-liquid base containing 1.6% freebase nicotine and tobacco flavouring |
| EVP-neat-TF | Neat e-liquid base containing 0% nicotine and tobacco flavouring |
| EVP-neat-PG/VG | Neat e-liquid base, 45:55 PG:VG only |
| EVP-bPBS-NS-TF | PBS-bubbled aerosol of e-liquid base containing 1.6% nicotine salt and tobacco flavouring |
| EVP-bPBS-FB-TF | PBS-bubbled aerosol of e-liquid base containing 1.6% freebase nicotine and tobacco flavouring |
| EVP-bPBS-TF | PBS-bubbled aerosol of e-liquid base containing 0% nicotine and tobacco flavouring |
| EVP-bPBS-PG/VG | PBS-bubbled aerosol of e-liquid base containing 45:55 PG:VG only |
| 1R6F-bPBS | PBS-bubbled smoke of the 1R6F reference cigarette |
| 1R6F-TPM | DMSO extract of 1R6F reference cigarette smoke trapped with a Cambridge filter pad |

Compositions of the neat e-liquids are found in [Table 2](#).

FB, freebase (nicotine); NS, nicotine salt; TF, tobacco flavouring.

Table 2. Formulations of neat e-liquids used in this study

| Test article | Content [w/w %] | | | |
|----------------|-----------------|----|----------|--------------------|
| | PG | VG | Nicotine | Tobacco flavouring |
| EVP-neat-TF | 41 | 55 | – | 4 |
| EVP-neat-FB-TF | 39.4 | 55 | 1.6 | 4 |
| EVP-neat-NS-TF | 39.4 | 55 | 1.6 | 4 |
| EVP-neat-PG/VG | 45 | 55 | – | – |

Values for PG do not include the 2.88% from flavouring.

FB, freebase (nicotine); NS, nicotine salt; TF, tobacco flavouring.

impingers each containing 10 ml of PBS solution (Sigma-Aldrich, Germany), resulting in a total sample stock volume of 30 ml. Cigarette smoke was generated using the Health Canada Intense smoking regime (bell-shaped puff profile, 55 ml puff volume, 2 s duration, 30-s intervals and 100% vent blocking; 54 puffs) to produce 1.8 puffs/ml PBS. EVP aerosol was generated using the Cooperation Centre for Scientific Research Relative to Tobacco Recommended Method No. 81 (square wave puff profile, 55 ml puff volume, 3 s duration, 30-s intervals; 120 puffs), to produce 4 puffs/ml PBS samples. Total particulate matter (TPM) samples were extracted as per the method described by Czekala *et al.* (28).

Test article composition

Nicotine and eight selected carbonyl (based on the list described by Buratto *et al.* (36)) levels within the freshly generated bPBS samples were quantified using liquid chromatography–tandem mass spectrometry (LC-MS/MS) (AB SCIEX API 6500 QTRAP) and high performance liquid chromatography with diode array detector (HPLC-DAD) (Agilent Technologies 1100 Series), respectively. Detection was carried out to internal Reemtsma SOPs (in accordance with ISO 17025 (37)). The internal standard for nicotine was

nicotine-d4, and 2,4-dinitrophenylhydrazine was used to identify carbonyls of interest. All reagents (including solvent used in the methods) were purchased from Sigma-Aldrich (Germany) unless stated otherwise.

Cell culture

C57/Bl6 B4418 wild-type mES cells were cultured in mES knock-out medium (Gibco) containing 10% foetal calf serum (FCS), 2 mM GlutaMAX, 1 mM sodium pyruvate, ×1 MEM non-essential amino acids, 100 mM β-mercaptoethanol and leukemia inhibitory factor (complete mES cell culture medium) and were propagated on irradiated primary mouse embryonic fibroblasts as feeders according to established protocols (9). For chemical exposure, cells were seeded 24 h prior to exposure on gelatine-coated plates in complete mES cell culture medium in the absence of feeder cells and, subsequently, exposed to the test compounds for 4 or 24 h.

Exposure

Stocks prepared in PBS, neat e-liquids and TPM extracted in dimethyl sulphoxide (DMSO) were added directly to the cell culture medium at concentrations detailed in [Supplementary Table S1](#) with a subsequent exposure period of 24 h (or 4 h for the ToxTracker-ACE assay). The following positive control substances were used in this study: aflatoxin B1 (CAS No.: 1162-65-8), benzo[*a*]pyrene (CAS No.: 50-32-8), cisplatin (CAS No.: 15663-27-1), diethyl maleate (CAS No.: 141-05-9), nicotine (CAS No.: 54-11-5), resorcinol (CAS No.: 108-46-3), taxol (CAS No.: 33069-62-4), tunicamycin (CAS No.: 11089-65-9) and vinblastine (CAS No.: 143-67-9). All tested compounds were purchased from Merck/Sigma-Aldrich. All compounds were dissolved in DMSO except cisplatin, which was dissolved in PBS, and final test concentrations in the cell culture medium are detailed in [Supplementary Table S1](#). Solvent concentration was the same in all wells and never exceeded 1% for DMSO.

Metabolic activation was included in the ToxTracker assay by the addition of S9 liver extract from Aroclor1254-induced rats (Moltox). For this, cells were exposed to the test samples in the presence of 0.25% S9 and required co-factors (RegenSysA+B, Moltox) for 24 h.

Endpoint data collection

ToxTracker analysis

ToxTracker analysis was performed as previously described (10). In brief, GFP-reporter expression was determined by flow cytometry using a MACSQuant X flow cytometer (Miltenyi Biotech), equipped with a 488-nm blue laser, 405-nm UV laser and an orbital shaker. Following 24 h of exposure, cells were washed with PBS, detached with trypsin and resuspended in PBS supplemented with 2% FCS, immediately followed by flow cytometry analysis. Reporter activity was determined by the mean fluorescence intensity of 10 000 intact cells. Cell numbers were determined using the absolute cell count that was performed during GFP detection by the flow cytometer. Experiments were performed in three independent biological replicates.

Cell cycle and polyploidy analysis

For the analysis of cell cycle progression following exposure of the ToxTracker cells, DNA staining was performed after 3.5 or 23.5 h of compound exposure. To stain the DNA, Hoechst 33342 was added to a final dilution of 1:500 in medium and cells were incubated with this for 30 min at 37°C. From here on, the cells were

protected from light. After incubation, cells were washed twice in PBS, detached using trypsin and resuspended in PBS + 2% FCS. Cells were then collected by centrifugation, resuspended and incubated for 10 min in extraction buffer, fixed by adding 25% glutaraldehyde to a final concentration of 0.5% and incubated for another 10 min. The fixative was quenched, and samples were then analysed using the MACSQuant X flow cytometer. For cell cycle and DNA content analysis of the cells, at least 10 000 single cells per exposure replicate were analysed by flow cytometry. Compound concentrations that induced >75% cell death in the ToxTracker cells after 24 h exposure were not considered in the ploidy and cell cycle analysis.

Endpoint data analysis

ToxTracker data were analysed using the ToxPlot software (10). Activation of a reporter cell line was considered positive when, at any applied dose, exposure to a compound resulted in >2-fold induction of GFP expression. This value was based on a weak positive effect threshold of 1.5-fold GFP induction, the signal increase that is at least five times higher than background (DMSO control) fluorescence standard deviation (10). The inferred statistical significance at this induction threshold is based on a confidence of >99.9% for positive induction at this value following extensive assay validation (9,10). Two-fold responses, therefore, were considered as defined, significant increases compared to control values. Measurements at concentrations that induced >75% cytotoxicity after 24-h of exposure were not considered for data analysis. The relative cell survival following a 24-h treatment was calculated as the ratio of the concentration of intact cells in treated populations to the concentration of intact cells in vehicle control treated populations, determined by the flow cytometer.

Cell cycle and aneuploidy analysis was performed using FlowLogic software (Inivai Technologies). For samples taken after 4-h exposures, the percentages of cells in G1, S and G2/M phases were quantified. For samples taken after 24 h of exposure, the percentage of cells with >4n DNA content (more DNA than the G2/M peak) was determined. A test article was classified as aneuploid when the percentage of aneuploid cells was above 4%, and an accumulation of cells in the G2/M phase of the cell cycle was observed after 4 h of treatment.

Results

Smoke/aerosol bPBS compositional analysis

In the bPBS samples generated with the EVP aerosols, the levels of the majority of carbonyls evaluated were either below the limit of quantification (LOQ) or were greatly reduced compared to those in the 1R6F smoke bPBS sample (Table 3). The 1R6F samples' smoke nicotine levels were consistent with historical data (data not shown), demonstrating reproducibility using analytical methodology. In contrast to the carbonyl data, in the nicotine-containing samples (freebase and nicotine salt), nicotine levels were comparable to those in the cigarette smoke bPBS. Low levels of nicotine were detected in the nicotine-free EVP aerosol bPBS (Table 3); however, no nicotine peak was recorded on the Certificate of Analysis generated for the e-liquids upon their production (Table 2). This is in line with the Association Française de Normalisation standard of defining an e-liquid as nicotine free if levels are <0.5 mg/ml (38). The nicotine detected in the nicotine-free aerosols was likely due to residual/background nicotine in the smoking/vaping machine tubing. An additional explanation could be a slight difference in sensitivity between analytical methods of e-liquids and PBS-trapped aerosols; however,

Table 3. Nicotine and carbonyl content ($\mu\text{g/ml}$ and $\mu\text{g/puff}$) of test article smoke or aerosols used in this study were measured using LC-MS/MS and HPLC-DAD, respectively

| Analyte | 1R6F-bPBS | | EVP-bPBS-TF | | EVP-bPBS-FB-TF | | EVP-bPBS-NS-TF | | EVP-bPBS-PG/VG | |
|---|------------------|--------------------|------------------|--------------------|------------------|--------------------|------------------|--------------------|------------------|--------------------|
| | $\mu\text{g/ml}$ | $\mu\text{g/puff}$ | $\mu\text{g/ml}$ | $\mu\text{g/puff}$ | $\mu\text{g/ml}$ | $\mu\text{g/puff}$ | $\mu\text{g/ml}$ | $\mu\text{g/puff}$ | $\mu\text{g/ml}$ | $\mu\text{g/puff}$ |
| Nicotine | 112.2 | 62.33 | <1 | <0.25 | 167 | 41.75 | 187 | 46.75 | <1 | <0.25 |
| Formaldehyde | 9.7 | 5.39 | 4.4 | 1.10 | 2.1 | 0.53 | 9.0 | 2.25 | 2.3 | 0.58 |
| Acetaldehyde | 167.3 | 92.94 | <LOQ | <LOQ | <LOQ | <LOQ | 3.3 | 0.83 | <LOQ | <LOQ |
| Acetone | 19.5 | 10.83 | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| Acrolein | 4.2 | 2.33 | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| Propionaldehyde | 8.7 | 4.83 | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| Crotonaldehyde | 4.4 | 2.44 | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| 2-Butanone (methyl ethyl ketone (butanone)) | 3.9 | 2.17 | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| <i>n</i> -Butyraldehyde | 3.5 | 1.94 | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |

The LOQ for nicotine was 0.01 $\mu\text{g/ml}$; the LOQ for formaldehyde was 0.25 $\mu\text{g/ml}$, acetaldehyde 1.5 $\mu\text{g/ml}$, acetone 1 $\mu\text{g/ml}$ and all other carbonyls 0.5 $\mu\text{g/ml}$. FB, freebase (nicotine); NS, nicotine salt; TF, tobacco flavouring.

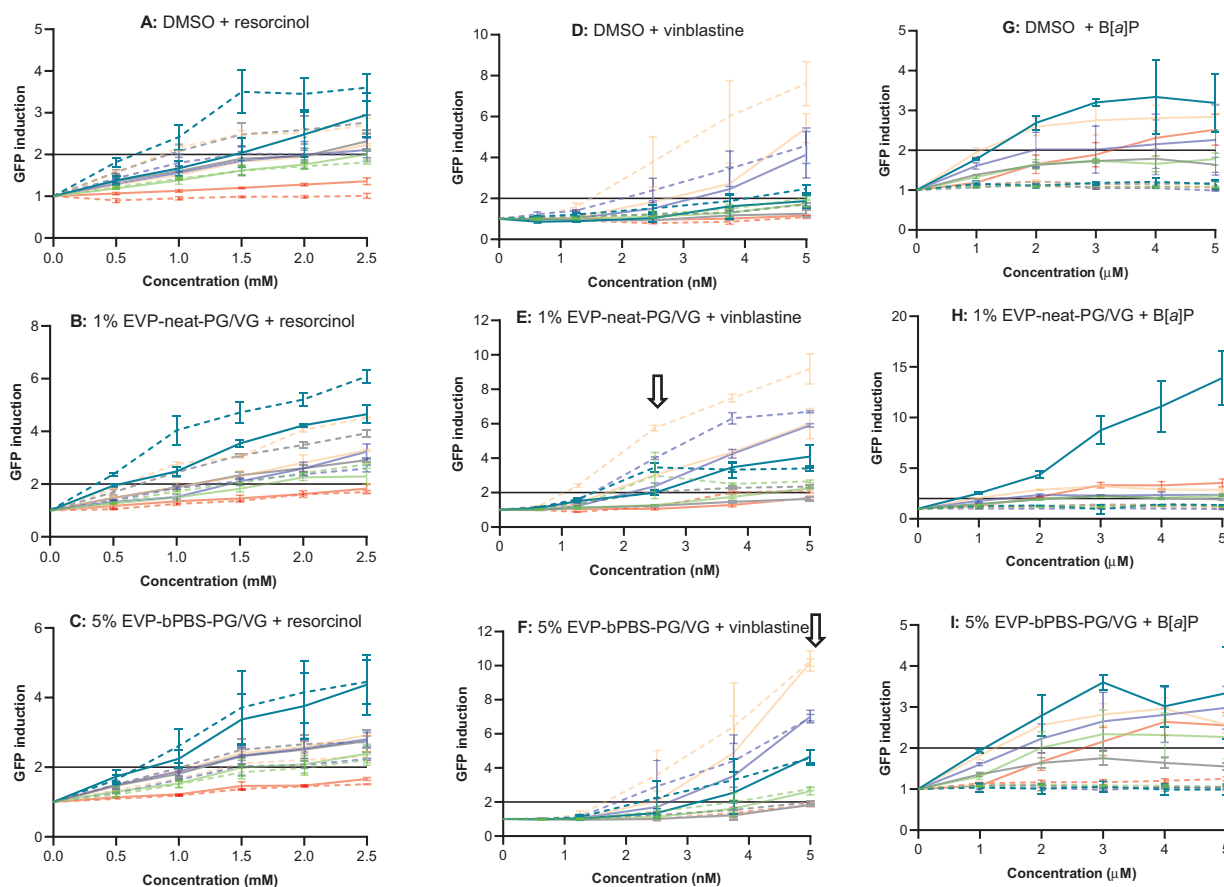


Fig. 1. GFP-reporter responses of six ToxTracker mES cell lines to resorcinol (A–C), vinblastine (D–F) and B[a]P (H–J) exposure (24 h) in the absence or presence of S9. Resorcinol, vinblastine or B[a]P were spiked into the cell culture in DMSO (concentration in cell culture medium 1%; A, D and G) within neat PG/VG (concentration in cell culture medium 1%; NEAT; B, E and H) or within PG/VG aerosol bPBS (concentration in cell culture medium 5%; C, F and I) to achieve final exposure concentrations detailed on the graphs (error bars indicate standard error of the mean; $n = 3$). The corresponding cell survival data can be found in [Supplementary Figure S2](#). Arrows indicate the concentration at which $\geq 75\%$ cytotoxicity was observed. For a key to the responses, see [Table 4](#).

the nicotine-free e-liquids from which the aerosols were generated still fall well within the limit for nicotine-free liquids. Formaldehyde levels in the EVP aerosol samples were elevated compared to the seven other carbonyls but did not exceed those measured in the 1R6F smoke-derived sample.

The detection limit in the ToxTracker-ACE assay was, in general, not greatly altered by the presence of test article vehicles

Three selected ECVAM-recommended positive control compounds (22) were spiked in DMSO, neat PG/VG or PG/VG aerosol bPBS to assess the effect of different vehicles on responses in the ToxTracker assay. Resorcinol-induced dose-dependent increases in induction of all of the endpoints to varying levels, with the strongest responses in the Srxn1 and BlvrB oxidative stress responses and the Rtkn DNA damage marker ([Figure 1A–C](#); [Table 5](#)). Although there were slight alterations in the induction doses, that is a difference of one test concentration ([Figure 1A–C](#); [Table 6](#)), these were not considered significant changes within the parameters of the assay. Additionally, there was no induction of aneuploidy using any of the vehicles, and cell cycle profiles induced by resorcinol were additionally unchanged with vehicle ([Supplementary Figures S5 and S6](#)).

Table 4. Key for the graphs in [Figures 1–7](#) and [Supplementary Figure S3](#)

| Endpoint | Marker |
|----------------------|--------------|
| DNA damage | --- Bsc12-S9 |
| | — Bsc12+S9 |
| | --- Rtkn-S9 |
| p53 activation | — Rtkn+S9 |
| | --- Btg2-S9 |
| Oxidative stress | — Btg2+S9 |
| | --- Srxn1-S9 |
| | — Srxn1+S9 |
| Protein damage | --- BlvrB-S9 |
| | — BlvrB+S9 |
| | --- Ddit3-S9 |
| — Ddit3+S9 | |
| — Positive induction | |

Solid lines indicate the presence of S9 and dashed lines indicate the absence of S9. The ToxTracker endpoint related to each marker is also detailed.

Again, in the presence of vinblastine, there were similar dose responses in the GFP endpoints ([Figure 1D–F](#)), and changes to the induction dose were not considered significant within the assay. The strongest responses (both +/-S9) were to the Rtkn DNA damage

response, Btg2 p53 activation and Srxn1 oxidative stress marker (Figure 1D–F; Table 5). Interestingly, whilst induction levels were largely unchanged, 75% cytotoxicity was observed at the top-tested dose of aqueous bubbled PG/VG (5%) plus vinblastine (5 nM) and at the even lower concentration of 1.25 nM vinblastine in the presence of neat PG/VG. Vinblastine, a known aneugen, induced increases in DNA content (24-h exposure) and additionally increases in cells in G2/M (4-h exposure) at higher doses in the presence of all test vehicles (Supplementary Figures S5 and S6).

Benzo[a]pyrene (B[a]P) induced dose-dependent increases in all of the six ToxTracker endpoints only following metabolic activation with S9 (Figure 1G–I), and the response profiles to all of the endpoints, excluding Srxn1, were very similar in the presence of the three delivery articles. However, in the presence of neat PG/VG, there was a large increase in induction of the Srxn1 response with dose to almost 5-fold at the top B[a]P dose of 5 µM (Figure 1H; Table 6). Cell cycling was not significantly different between changes in vehicle (Supplementary Figure S5) and additionally no increases in DNA content were observed (Supplementary Figure S6).

1R6F smoke bPBS induced strong responses in the ToxTracker endpoints, whereas EVP aerosol bPBS did not

Following dose-range finding experiments (data not shown), five test concentrations (plus a negative control) were selected for the testing of ToxTracker endpoint induction. Tests were carried out alongside treatment with positive reference compounds (Supplementary Table S1).

Up to a maximum concentration of 10% bPBS in the cell culture medium, +/-S9, the three flavoured EVP aerosol bPBS samples did not induce any response in any of the GFP endpoints, and there was no reduction in cell viability (Figures 2A and B and 3A; Supplementary Figure S2). The PG/VG bPBS was tested up to 5% (tested at a concentration to match that used in the positive control spiking experiments). Up to this concentration, the test article did not induce any response in any of the endpoints (Figure 3B), and there was no reduction in cell viability, +/-S9 (Supplementary Figure S2).

The 1R6F reference cigarette smoke bPBS induced a decrease in cell survival with increasing dose, with 50% cytotoxicity observed at a test concentration of 8% bPBS (Supplementary Figure S2). Additionally, with increasing concentration, Rtkn-GFP, Btg2-GFP, Srxn1-GFP and BlvrB-GFP were activated (>2-fold GFP induction). However, there was no induction above the threshold in Bcl2-GFP or Ddit3-GFP (both +/-S9; Figure 4A). There was a sharp decrease in the Srxn1-GFP signal between 5% and 10% in both the presence and absence of S9. As there was more than 2-fold induction of the Rtkn-GFP DNA damage reporter, the 1R6F bPBS sample was classified as genotoxic, whereas the EVP aerosol bPBS samples were not.

Neat flavoured e-liquids induced the ToxTracker oxidative stress markers, Srxn1 and BlvrB

The three flavoured e-liquids tested induced ~10–15% decreases in cell survival relative to the negative control treatments at the top dose (1%; Supplementary Figure S2) and there was no decrease in cell survival relative to the control following exposure to neat PG/VG. Additionally, exposure to neat PG/VG did not induce an increase in any of the six GFP markers (Figure 6B). The three flavoured neat e-liquids induced dose-dependent increases in both the Srxn1-GFP and BlvrB-GFP markers. These responses were amplified in the presence of S9, with the response to the nicotine-free flavour below the 2-fold induction

Table 5. Fold change induction of the ToxTracker GFP reporters following cell exposure (24 h) to the maximum tested concentrations of resorcinol (2.5 mM), vinblastine (5 nM) and B[a]P (5 µM) in DMSO (concentration in cell culture medium 1%), within neat PG/VG (concentration in cell culture medium 1%) or within PG/VG aerosol bPBS (concentration in cell culture medium 5%)

| | Fold change GFP induction | | | | | | | | | | | |
|---------------------------------------|---------------------------|------|-------|-------|------|------|-------|-------|-------|------|-------|------|
| | Bcl2 | | Rtkn | | Btg2 | | Srxn1 | | BlvrB | | Ddit3 | |
| | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 |
| DMSO + 2.5 mM resorcinol | 1.81 | 2.00 | 2.70 | 2.23 | 2.00 | 2.11 | 3.60 | 2.95 | 2.77 | 2.32 | 1.01 | 1.36 |
| 1% EVP-neat-PG/VG + 2.5 mM resorcinol | 2.75 | 2.29 | 4.52 | 3.28 | 2.59 | 3.23 | 6.08 | 4.65 | 3.93 | 2.91 | 1.70 | 1.83 |
| 5% EVP-bPBS-PG/VG + 2.5 mM resorcinol | 2.21 | 2.39 | 2.32 | 2.92 | 2.23 | 2.80 | 4.45 | 4.37 | 2.72 | 2.75 | 1.51 | 1.66 |
| DMSO + 5nM vinblastine | 2.01 | 1.74 | 7.61 | 5.44 | 4.58 | 4.14 | 2.47 | 1.87 | 1.71 | 1.25 | 1.09 | 1.16 |
| 1% EVP-neat-PG/VG + 5 nM vinblastine | 2.65 | 2.25 | 9.18 | 6.00 | 6.69 | 5.90 | 3.40 | 4.10 | 2.35 | 1.63 | 2.12 | 1.69 |
| 5% EVP-bPBS-PG/VG + 5 nM vinblastine | 2.77 | 2.63 | 10.27 | 10.18 | 6.94 | 7.02 | 4.58 | 4.67 | 1.98 | 1.82 | 1.85 | 1.85 |
| DMSO + 5µM B[a]P | 1.09 | 1.78 | 1.17 | 2.84 | 0.99 | 2.26 | 1.15 | 3.19 | 1.17 | 1.64 | 1.07 | 2.52 |
| 1% EVP-neat-PG/VG + 5 µM B[a]P | 1.25 | 2.32 | 1.27 | 2.86 | 0.97 | 2.38 | 1.35 | 13.91 | 1.29 | 1.92 | 1.21 | 3.54 |
| 5% EVP-bPBS-PG/VG + 5 µM B[a]P | 1.06 | 2.27 | 0.99 | 2.57 | 1.04 | 2.98 | 0.99 | 3.34 | 1.04 | 1.55 | 1.25 | 2.55 |

Red cells indicate an induction >2-fold that of negative control levels, orange cells indicate >1.5-fold induction (weak induction) and green cells indicate <1.5-fold (no induction).

threshold (Figures 5A and B and 6A). Weak induction of p53-related Btg2 was observed following exposure to the two nicotine-containing flavours; however, these responses also did not exceed the threshold of a 2-fold change relative to the control (Figures 5A and B).

TPM induced strong responses in the ToxTracker assay

TPM (in DMSO) induced a decrease in cell survival with increasing concentration (Supplementary Figure S2). In both the absence and presence of S9, 50% cytotoxicity was observed at a test concentration of around 0.7% (Supplementary Figure S2). Activation of p53-associated Btg2-GFP, oxidative stress markers Srxn1-GFP and Blvrb-GFP and UPR marker, Ddit3-GFP, was observed in both the absence and presence of S9 (Figure 4B). In the absence of metabolism, there was an increase in induction of Bsl2-GFP and Rtkn-GFP; however, this induction was weak (>1.5- but <2-fold induction) in the presence of S9.

Nicotine induced an oxidative stress response at a supraphysiological concentration only

Upon exposure to up to 10 mM nicotine (+/-S9), the Srxn1 oxidative stress response was observed at the top dose only (Figure 7). This correlated with a decline in cell survival of approximately 60% (Supplementary Figure S2). The four positive control compounds tested alongside the treatments in this part of the study induced GFP responses and cytotoxicity profiles (Supplementary Figures S2 and S3) consistent with Toxys's historical control data (data not shown).

Flow cytometry data obtained from the ToxTracker assay was run through the ToxPlot software to inform on effects relative to test article potency (Supplementary Figure S4). The four aqueous bubbled EVP aerosols did not induce any increases in induction of GFP responses or cytotoxicity up to the maximal concentrations tested. However, GFP responses to the neat e-liquids and 1R6F-derived samples increased with increasing cytotoxicity (Supplementary Figure S4).

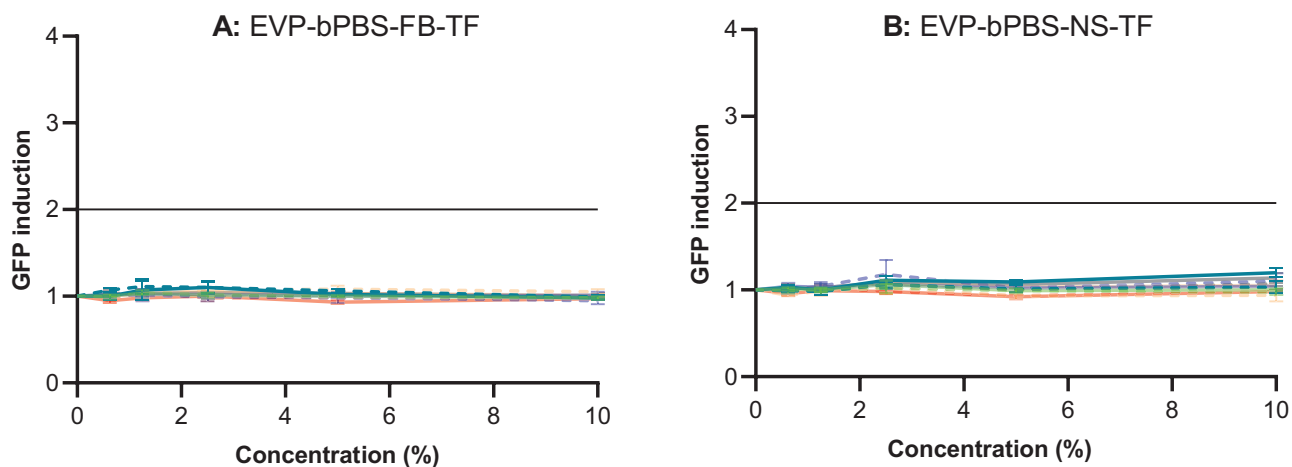


Fig. 2. Responses of the six ToxTracker cell lines to EVP aerosol bPBS, added at concentrations up to 10% (0%, 0.6%, 1.3%, 2.5%, 5% and 10%) in the cell culture medium, in the presence or absence of S9, for 24 h. (A) Aerosol bPBS of e-liquid containing 1.6% freebase nicotine, tobacco flavour. (B) Aerosol bPBS of e-liquid containing 1.6% nicotine salt, tobacco flavour (error bars represent standard error of the mean; $n = 3$). For a key to the responses, see Table 4. The corresponding cell survival data can be found in Supplementary Figure S2.

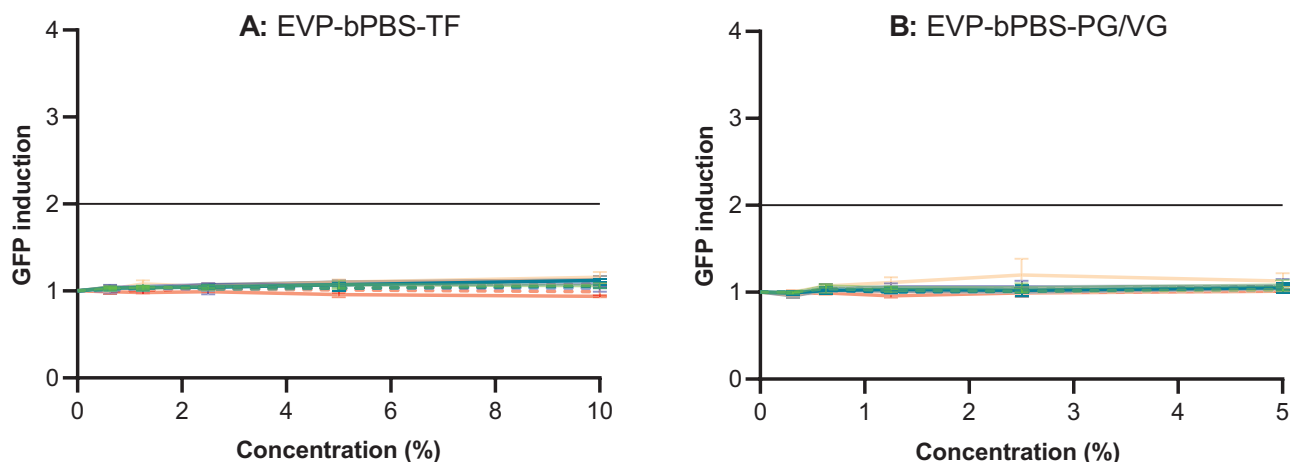


Fig. 3. Responses of the six ToxTracker cell lines to EVP aerosol bPBS in the cell culture medium, in the presence or absence of S9, for 24 h. (A) Aerosol bPBS of tobacco flavour e-liquid containing 0% nicotine (concentrations of 0%, 0.6%, 1.3%, 2.5%, 5% and 10%). (B) Aerosol bPBS of e-liquid base (PG/VG; error bars represent standard error of the mean; $n = 3$). For a key to the responses, see Table 4. The corresponding cell survival data can be found in Supplementary Figure S2.

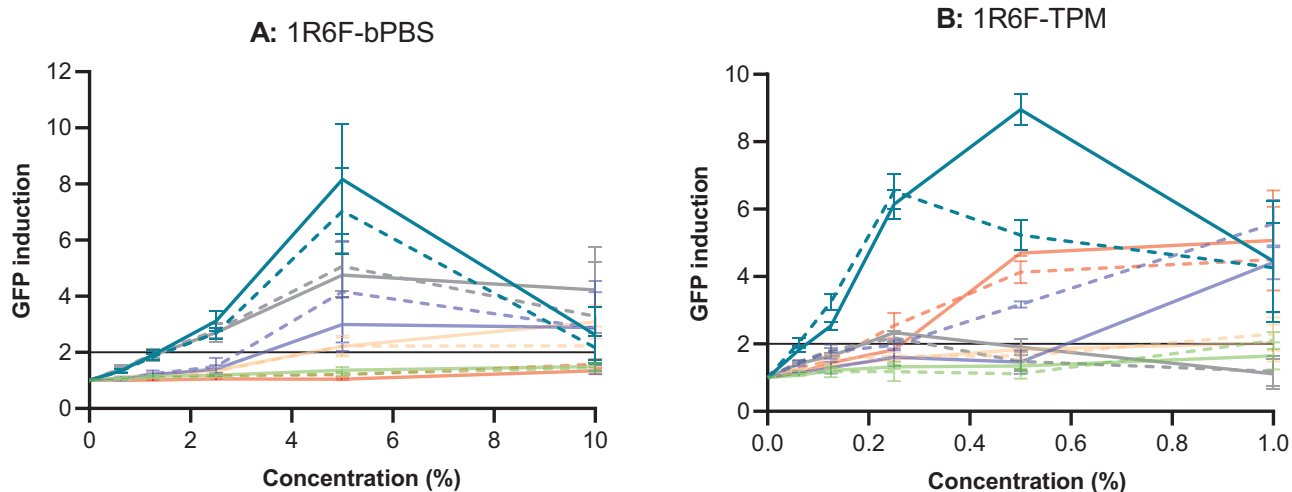


Fig. 4. Responses of the six ToxTracker cell lines to (A) 1R6F smoke bPBS, added at concentrations up to 10% (0%, 0.6%, 1.3%, 2.5%, 5% and 10%) in the cell culture medium; (B) 1R6F smoke TPM, tested up to 1% (vehicle DMSO; 0%, 0.06%, 0.13%, 0.25%, 0.5% and 1%) in the cell culture medium. Exposures were carried out in the presence or absence of S9 for 24 h (error bars represent standard error of the mean; $n = 3$). For a key to the responses, see Table 4. The corresponding cell survival data can be found in Supplementary Figure S2.

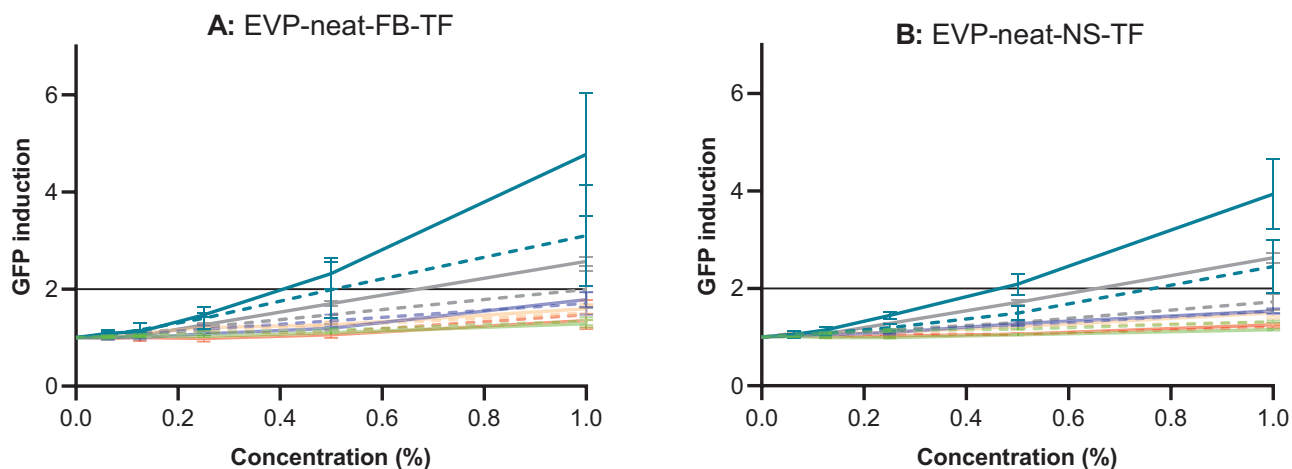


Fig. 5. Responses of the six ToxTracker cell lines to neat e-liquid added to the cell culture medium at test concentrations up to 1% (0%, 0.06%, 0.13%, 0.25%, 0.5% and 1%), in the absence or presence of S9, for 24 h. (A) Neat e-liquid containing 1.6% freebase nicotine, tobacco flavour. (B) Neat e-liquid containing 1.6% nicotine salt, tobacco flavour (error bars represent standard error of the mean; $n = 3$). For a key to the responses, see Table 4. The corresponding cell survival data can be found in Supplementary Figure S2.

1R6F aqueous bubbled smoke and TPM did not induce aneugenic responses in the ToxTracker-ACE assay

Due to the strong responses induced by the 1R6F-derived samples, these were further investigated with the ToxTracker-ACE extension. The aqueous bubbled 1R6F smoke samples induced a small increase in cells in G2/M following 4-h exposure at the top-tested concentration of 10%; however, small dose-dependent decreases in the number of cells in G2/M were observed following TPM exposure (Supplementary Figure S7). Neither of the cigarette smoke-derived samples induced increases in DNA content following 24-h exposure (Supplementary Figure S8). In combination, the data from these two endpoints suggests a non-aneugenic mode of action for both test articles.

The GFP responses obtained for each article tested were tabulated to indicate the fold change induction of the reporters at 10%, 25%, 50% and 75% cytotoxicity (Supplementary Table S3). Cytotoxicity

LC50 values were also collated along with the no-observed-effect level, lowest-observed-effect level, no-observed-genotoxic-effect level and lowest-observed-genotoxic-effect level (Supplementary Table S4). In summary, the results suggest that the 1R6F samples were genotoxic; however, the EVP-derived samples were not.

Discussion

This study aimed to assess the effects of EVP samples, either in the form of neat e-liquids or aerosol bPBS, in the ToxTracker assay, compared to those of Kentucky 1R6F reference cigarette smoke samples.

Assessment of the detection limits of ToxTracker/ACE using positive control compounds

The compositionally complex nature of cigarette smoke and EVP liquids and aerosols is a key characteristic of the test samples used here, and the synergistic effects of the components within them is

Table 6. Concentrations of resorcinol, vinblastine and B[a]P [in DMSO, neat PG/VG (1% concentration in the cell culture medium) or PG/VG aerosol bPBS (5% concentration in the cell culture medium)] that induced significant responses of ToxTracker DNA damage reporters, Bsc12 and Rtkn, following 24-h exposure in either the absence or presence of S9

| | Bsc12 | | Rtkn | | Aneuploidy | |
|---------------------------------|--------|-----------|---------|-----------|------------|---------|
| | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 |
| DMSO + resorcinol | - | 2.5 mM | 1 mM | 2.5 mM | - | - |
| 1% EVP-neat-PG/VG + resorcinol | 1.5 mM | 2 mM | 1 mM | 1.5 mM | - | - |
| 5% EVP-bPBS-PG/VG + resorcinol | 2.5 mM | 2 mM | 1.5 mM | 1.5 mM | - | - |
| DMSO + vinblastine | 5 nM | - | 2.5 nM | 3.75 nM | 1.25 nM | 1.25 nM |
| 1% EVP-neat-PG/VG + vinblastine | - | - | 1.25 nM | 2.5 nM | 2.5 nM | 1.25 nM |
| 5% EVP-bPBS-PG/VG + vinblastine | 5 nM | 5 nM | 2.5 nM | 2.5 nM | 3.75 nM | 2.5 nM |
| DMSO + B[a]P | - | - | - | 2 μ M | - | - |
| 1% EVP-neat-PG/VG + B[a]P | - | 3 μ M | - | 1 μ M | - | - |
| 5% EVP-bPBS-PG/VG + B[a]P | - | 2 μ M | - | 2 μ M | - | - |

Concentrations that resulted in >4n DNA content (aneuploidy) in cells following 24-h exposure in mES cells (in the absence or presence of S9) are also expressed. Dashes represent an absence of response to the tested concentrations.

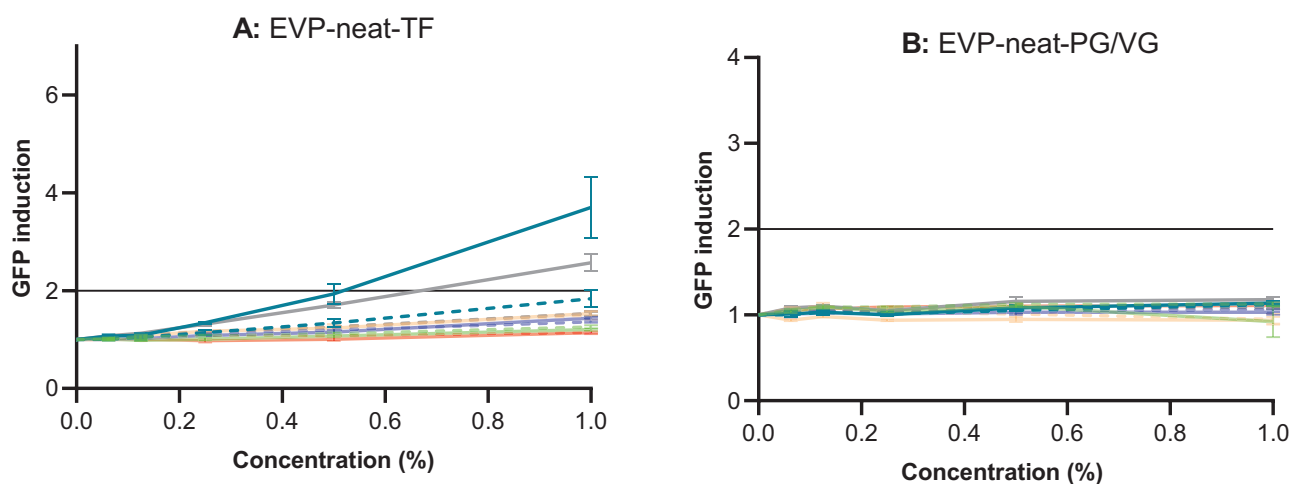


Fig. 6. Responses of the six ToxTracker cell lines to neat e-liquid added to the cell culture medium at test concentrations up to 1% (0%, 0.06%, 0.13%, 0.25%, 0.5% and 1%), in the absence or presence of S9, for 24 h. (A) Neat e-liquid containing tobacco flavouring, 0% nicotine. (B) Neat unflavoured PG/VG, 0% nicotine (error bars represent standard error of the mean; $n = 3$). For a key to the responses, see Table 4. The corresponding cell survival data can be found in Supplementary Figure S2.

an important consideration. Vinblastine, an inhibitor of microtubule polymerisation, is a known aneugen (22,39). A combination of induction of the Rtkn DNA damage reporter and aneuploidy, which indicates an aneugenic mode of action, was demonstrated in the range of 1.25–3.75 nM vinblastine when assessed in the presence of all three test vehicles. However, in the presence of 1% neat PG/VG, the exposure to vinblastine was more cytotoxic than in the presence of bPBS or DMSO. The cytotoxicity threshold (>75%) was reached with a neat PG/VG concentration of 1% combined with 1.25 nM vinblastine, whilst a combination of 5% bPBS or 1% DMSO and the top-tested vinblastine concentration of 5 nM reached the same level of cytotoxicity. The cytotoxicity profiles of vinblastine +/-S9 were similar. The shift in response in the presence of neat e-liquids may be due to osmolarity increases within the cell cultures causing increased cellular sensitivity to the already potent vinblastine.

The xenobiotic response to B[a]P exposure induces a number of metabolic products and these can include reactive oxygen species (ROS) and other DNA reactive species (40,41). As one of the main (pro-)carcinogens present following cigarette combustion,

it is unsurprising that B[a]P induced all of the ToxTracker endpoints, in a dose-dependent manner, in the presence of S9 only. These response profiles closely resembled each other following exposure of cells to B[a]P in DMSO and PG/VG (5%; Figure 1G–I). However, in the presence of neat PG/VG, there was a sharp increase in the Srxn1 oxidative stress reporter with dose (Figure 1H). As observed, the presence of the neat e-liquid and the presence of B[a]P induced this reporter (Figures 1G, 5A and B and 6A) and, therefore, this may be due to an additive effect. With a focus on the DNA damage response (Rtkn and Bsc12) in the presence of the three vehicles, B[a]P induced these within the range of 1–3 μ M, and induction levels were similar. It can, therefore, be concluded that the different vehicles did not greatly alter the sensitivity of the assay here.

Exposure to resorcinol-induced dose-dependent increases in the ToxTracker GFP endpoints, and these responses were not greatly altered in the presence of the three vehicles (Figure 1A–C; Table 6). Although resorcinol has been widely reported as negative for genotoxicity and carcinogenicity, with positive responses associated

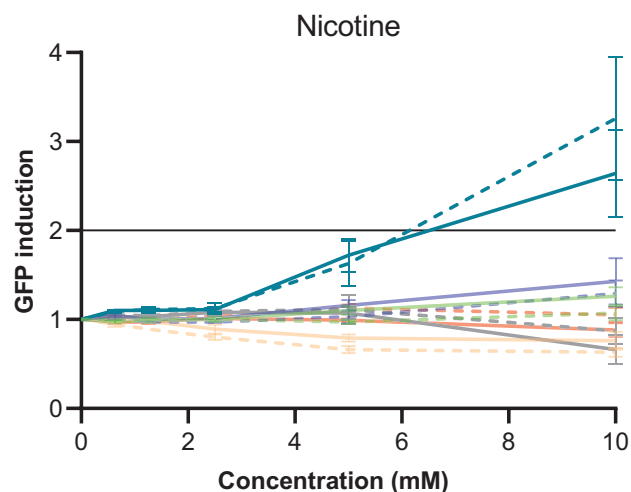


Fig. 7. Responses of the six ToxTracker cell lines to nicotine, tested up to 10 mM (vehicle DMSO; 0, 0.625, 1.25, 2.5, 5 and 10 mM) in the cell culture medium, in the absence or presence of S9, for 24 h (error bars represent standard error of the mean; $n = 3$). For a key to the responses, see Table 4. The corresponding cell survival data can be found in Supplementary Figure S2.

with p53 deficiency (22), it is interesting to note that there were slight increases with both of the DNA damage reporters, Rtnk and Bsc12, in addition to the p53-associated Btg2 marker, with dose. This was coupled with increases in oxidative stress reporters, indicating that the responses observed here were possibly due to ROS. Furthermore, the activation of the DNA damage markers was observed at test concentrations of 1 mM or higher. The results reported here are not consistent with the results reported by Hendriks *et al.* (10) in the validation study of the extended ToxTracker assay as they did not observe the induction of Rtnk and Bsc12 following exposure to resorcinol. Dose-range finding experiments for the present study, to induce observable responses, indicated that a concentration of 2.5 mM would cause ~40% cytotoxicity (Supplementary Figure S1A), and this was selected as a maximum test concentration. The study by Hendriks *et al.* (10) only tested resorcinol up to a concentration of 1 mM; however, it still had slightly greater potency at this concentration in the present study, perhaps due to the batch of chemical used. Resorcinol has been shown to induce contradictory results in the *in vitro* micronucleus test upon comparison of human-derived TK6 cells and V79 rodent cells (42).

Determination of testing concentrations for neat e-liquids: neat e-liquids induced an oxidative stress response at high concentrations

Based on a preliminary assessment with the ToxTracker cells (data not shown), neat e-liquids were determined to be suitable to test at concentrations up to 1% in the cell culture medium in this study. PG and VG are commonly used in consumer products, such as cosmetics and foodstuffs, and are not considered toxic in these formats. However, potential effects on inhalation, such as minor respiratory tract irritation, are still subject to investigation (43). Increased effects (induction of oxidative stress reporters) were observed with metabolism in the case of (tobacco) flavoured e-liquids and not the unflavoured PG/VG, indicating that the metabolism of flavours in the e-liquid may play a role in the responses observed. However, it is

important to note that the observed effect was only detected with the neat e-liquid, which is not consumed by the (adult) smoker, and was included in this study to screen for effects of different e-liquid compositional combinations. No increase in effect was observed with any of the flavoured EVP aerosol samples, indicating that the metabolism of aerosol components in the samples tested does not have a (geno)toxic effect.

Increased solution osmolarity with increasing PG/VG concentration has been widely reported in a number of cell lines exposed to e-liquids, postulated to be due to deficient hyperosmotic clearance in *in vitro* cultures (26–28). Similarly, osmolality is a consideration highlighted in Organisation for Economic Co-operation and Development guidelines (44). Gonzalez-Suarez *et al.* (26) reported that higher concentrations of PG/VG, and particularly PG, *in vitro*, increased solution osmolarity and, therefore, cytotoxicity via hyperosmotic stress. However, osmolarity was not reported to be affected by nicotine (26), consistent with our comparison of osmolarity of cell culture medium containing nicotine-free or nicotine-containing neat e-liquids (Supplementary Table S2). Osmotic stress has also been associated with oxidative stress response, DNA damage and eventual cell death (45), which is consistent with the activation of the oxidative damage reporters in the ToxTracker assay. However, the induction of the DNA damage reporters was not observed in this study, suggesting that the neat e-liquid samples tested are not (directly) genotoxic. The absence of induction of the DNA damage reporters Rtnk and Bsc12 here may indicate that, in studies reporting DNA damage caused by osmotic changes *in vitro*, genotoxicity may be due to secondary effects through oxidative damage.

The screening of neat e-liquids is, however, a valuable step to assess the effects of combinations of ingredients. Its application to *in vitro* cell cultures is quick and of relative experimental simplicity (32) and, as this study has demonstrated, can indicate effects that could subsequently be investigated for the corresponding aerosol. Applying more EVP flavours in the ToxTracker assay would be the next step in further validation of this. In light of the findings on effects of osmolarity in the ToxTracker system, neat e-liquid testing would be suitable up to a concentration 1% in the cell culture medium.

Analysis of bPBS composition and limitations of the bPBS exposure approach

The bubbling of smoke/aerosol through an aqueous solution (e.g. PBS, cell culture medium) to achieve *in vitro* exposure has been used in various studies (27,30,46–48). This trapping method accounts for the water-soluble fraction of the smoke/aerosol, which is thought to be representative of the fraction soluble in the blood (47) and also allows a means of exposure to cells that must be submerged in medium. This is a limitation of the ToxTracker assay, that is, the mES cells cannot be exposed to whole smoke/aerosol, which would be achieved at the air-liquid interface and, therefore, the EVP aerosol bPBS exposures tested in the study only considered this water-soluble fraction of EVP aerosol. This is also a limitation of the bPBS exposure approach as it is not representative of lipophilic harmful and potentially harmful constituents (HPHCs) within the smoke/aerosol. As the 1R6F reference cigarette smoke TPM fraction tested, a more lipophilic fraction, filter trapped and extracted in DMSO, induced an altered response compared to the 1R6F smoke bPBS extract, it would be interesting in the future to apply the equivalent, aerosol

collected mass, even in combination with the aerosol bPBS, from EVP aerosol to the ToxTracker assay.

Measurement of levels of selected analytes within the bPBS was carried out to confirm trapping efficiency of smoke/aerosol within the PBS. The eight carbonyls analysed were selected based on the list analysed by Buratto *et al.* (36), representing some of the major carbonyls present in HPHC lists of regulatory bodies, including the Food and Drug Administration and Health Canada (49,50). The reduced, or below-LOQ, levels of carbonyls per puff in the EVP aerosol bPBS, compared to smoke bPBS, recorded in this study may explain the reduced effects of EVP aerosol bPBS compared to 1R6F cigarette smoke bPBS in the ToxTracker assay. Reduced HPHC, such as carbonyl, levels in EVP aerosol compared to cigarette smoke are thought to contribute to the reduced harm potential of EVPs (31,51). There were detectable levels of formaldehyde in the EVP aerosol bPBS, with particularly increased levels in the nicotine salt sample. However, this was still at around 2-fold lower per puff than in the cigarette smoke sample, and these levels were not high enough to induce any cellular stress responses to EVP aerosol bPBS in comparison to 1R6F smoke bPBS.

No (geno)toxic response to EVP aerosol bPBS was observed

At the bPBS concentrations tested, there was no observed reduction in cell viability and no inductive effects in the six ToxTracker GFP endpoints. Additionally, no response was observed in this study in either the absence or presence of S9, indicating that the EVP aerosol extracts neither contained toxicants nor pro-toxicants at levels that would induce cellular stress. In this study, the ToxTracker assay indicated that the EVP bPBS test articles (and the e-liquids) were not genotoxic.

There is a body of research into the potential genotoxic and mutagenic effects of EVP aerosols (extracts and whole) both *in vivo* and *in vitro*. Wiczorek *et al.* (29) demonstrated that the whole aerosols, and neat e-liquids, of a range of EVP flavours did not cause genotoxicity (micronucleus assay) or mutagenicity (Ames test) under the conditions tested. Further to this, Rudd *et al.* (31) correlated the reduction in EVP aerosol chemical compositional complexity with a lack of genotoxic and mutagenic responses to the *myblu*TM tobacco-flavoured (1.6% nicotine) whole aerosol, consistent with the lack of genotoxicity markers in the current study. Such reduction in effects (compared to those of cigarette smoke samples) also correlates with the observations of a number of previous studies on EVP aerosols in a variety of toxicological endpoints and even those testing at extremely high exposure levels (4,52–55). There are, however, studies that demonstrate the genotoxic and mutagenic potential of EVP aerosols both *in vitro* and *in vivo* (55–60). Ganapathy *et al.* (57) found that, in human cells, DNA damage, including oxidative damage, following chronic exposures to e-aerosols was potentially attributed to impaired DNA repair, and Lee *et al.* (58) also observed reduced repair capacity in vapour-exposed mice's lungs and human cell lines. DNA repair evaluation was not within the scope of this study; however, a lack of induction of any of the GFP stress-related/ initial DNA damage reporters indicates that such repair responses would not be relevant to the aerosol bPBS test articles used here. Oxidative stress was commonly found to play a role in the observed DNA damage in these studies (57,59,60) in contrast to the lack of oxidative stress induced by the aerosol fraction in the EVP bPBS samples in the current study. However, the variety of different products, with varying e-liquid compositions and device designs and the different extracts/

exposure methodologies and timeframes used makes it difficult to directly compare the results of all of these studies.

Mechanisms of cellular responses to PBS-trapped cigarette smoke and filter-trapped TPM

Large increases in the oxidative stress reporters, *Srxn1* and *Blvrb*, were observed following exposure to 1R6F smoke bPBS. Cigarette smoking has indeed been widely associated with increased oxidative stress both *in vitro* and *in vivo*, and this is implicated in a number of smoking-associated pathologies, including cardiovascular disease, chronic obstructive pulmonary disease and lung cancer (52,63,64). At a test concentration of 10%, the Nrf2-associated *Srxn1* signal decreased, correlating with a decrease in cell survival. This could be due to excessive oxidative stress burden, which can subsequently lead to cell death (28,63,65). The induction of stress responder, p53-associated *Btg2-GFP* and the *Rtkn* DNA damage reporter is consistent with a body of evidence on the known genotoxicity of cigarette smoke constituents (53,66,67). The induction of such responses within the ToxTracker assay illustrates its sensitivity as previous studies have not detected responses to aqueous bubbled cigarette smoke within the limits of cytotoxicity measures (68).

As with exposure to the 1R6F smoke bPBS, there was an initial increase in the induction of the *Srxn1* reporter with dose followed by a decline. A decline in ROS has been observed following exposure of (normal human bronchial epithelial (NBHBE)) cells to TPM *in vitro*, where effects may be both time and pathway dependent (28). Differences observed in the mechanisms between TPM and smoke bPBS is unsurprising as the mixture of components differs between the two (69). As mentioned earlier, this could be due to the difference in the fractions of cigarette smoke trapped in an aqueous medium (PBS) and DMSO. As cigarette smoking is known to induce a number of (geno)toxic endpoints (29,31,66,70), the induction of the p53 response is unsurprising. This suggests that near basal p53 levels may be sufficient in the initial cellular response to the 1R6F-derived samples (71). Furthermore, activation of *Ddit3*, involved in the UPR, including endoplasmic reticulum stress, cell cycle arrest and apoptosis, may be caused by the increased oxidative stress-induced protein damage within the system. Both the p53 and UPR response can lead to apoptosis (19,72), which is consistent with a reduction in cell survival with increasing TPM concentration. In concordance with this, cigarette smoke extracts have been previously demonstrated to cause apoptosis (73).

Cigarette-derived samples did not exhibit aneugenic mechanisms in the extended ToxTracker-ACE assay

Although toxicity of cigarette smoke and TPM occurs via many mechanisms, there is evidence that these include aneugenic actions (74,75). The selective activation of *Rtkn*, cell cycle arrest at G2/M and polyploidy can indicate an aneugenic mode of action (10). Whilst there was weak, dose-dependent activation of *Rtkn* following exposure to the 1R6F-derived samples, there were no increases in DNA content (aneuploidy) following exposure to these samples (Supplementary Figure S8). Additionally, whilst there was a small increase in cells in the G2/M phase of the cell cycle at the top dose of smoke bPBS, this effect was not recorded in the cell populations exposed to TPM (Supplementary Figure S7). Whilst these findings indicate that aneugenicity is not in the initial cellular response to cigarette smoke, with likely mechanisms associated with oxidative stress, this assay applied short-term exposures (24 h for GFP induction and polyploidy assessment and 4 h for cell

cycle analysis) with a limited exposure dose range. Therefore, repeated or prolonged exposures to cigarette smoke may reveal secondary or cumulative toxicological responses, but further experimentation for investigation into this would be required.

Effects of nicotine and relative exposure

Nicotine levels are often used as an indication of exposure in the testing of cigarette- and NGP-derived samples (28,76,77). However, nicotine has also been extensively characterised for its toxicological action both *in vitro* and *in vivo* and has been observed to induce genotoxic outcomes, including chromosome damage, in addition to oxidative stress (3,26,34,78–81). However, these effective concentrations are often at supraphysiological levels (3,28,82) (measured physiological nicotine levels range from 10 to 37 ng/ml (83)) and, therefore, have limited physiological relevance.

When nicotine (in DMSO) was applied to the ToxTracker assay, positive induction (in the Srxn1 oxidative stress response only) was only observed at the top-tested concentration of 10 mM (1622.3 µg/ml), a level that greatly exceeds physiologically relevant levels. This concentration was also 100-fold higher than the top concentrations of nicotine in the aqueous bubbled EVP aerosol and cigarette smoke samples and 10-fold higher than those in the neat sample. In light of these findings, the responses observed to the e-liquid and cigarette-derived samples are not thought to be driven by the presence of nicotine as the concentrations were at levels below those of toxicological effect. This is also consistent with the findings of Farsalinos *et al.* (52) and Leigh *et al.* (84), who found that the presence of nicotine in e-liquid samples did not affect cell viability *in vitro*. In an *in vivo* study by Panitz *et al.* (85), it was found that oxidative stress caused in nematodes upon exposure to a variety of test e-liquids (and their aerosols) was not nicotine dependent, despite greater effects observed across a number of endpoints to nicotine-containing liquids. It is, therefore, perhaps more informative to examine the effects of the whole mixtures as this most closely represents their compositional interactions. As expected, the presence of either free-base or nicotine salt in the EVP samples (neat or bPBS) did not induce significant differences to each other in the responses of the six ToxTracker cell lines.

Conclusions

This study aimed to screen a selection of EVP samples for potential genotoxicity, including mechanistic insights, and compare them to those observed in 1R6F reference cigarette samples in the ToxTracker assay. EVP liquid base components, PG and VG, were found not to interfere with the effects of positive control compounds, which enables future screening of additional e-liquid ingredients (i.e. flavourings) in these complex mixtures. The tobacco-flavoured and nicotine-containing e-liquids tested did not cause genotoxic effects in the ToxTracker assay, neat or as bPBS aerosol extracts. However, testing would only be suitable at levels ≤1% neat e-liquid in the cell culture medium to ensure that effects on solution osmolarity are not present. Overall, the assay has the potential for future application in the assessment battery for complex mixtures from NGPs, including EVPs, in combination with already established regulatory assays, by providing the additional benefit of insights into (geno) toxic mechanisms.

Supplementary data

Supplementary data are available at *Mutagenesis* Online.

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