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RESEARCH ARTICLE

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Characterisation of a smoke/ aerosol exposure in vitro system (SAEIVS) for delivery of complex mixtures directly to cells at the air-liquid interface

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Abstract

In vitro testing is important to characterise biological effects of consumer products, including nicotine delivery products such as cigarettes, e-cigarettes and heated tobacco products. Users' cells are exposed to these products' aerosols, of variant chemical compositions, as they move along the respiratory tract. In vitro exposure systems are available to model such exposures, including delivery of whole aerosols to cells, and at the air-liquid interface. Whilst there are clear advantages of such systems, factors including time to aerosol delivery, aerosol losses and number of cell cultures that can be exposed at one time could be improved. This study aimed to characterise a custom-built smoke/ aerosol exposure in vitro system (SAEIVS) using 1R6F reference cigarette smoke. This system contains five parallel smoking chambers and delivers different dilutions of smoke/ aerosol to two separate cell culture exposure chambers in <10 s. Using two dosimetry measures (optical density 400 nm [OD^{400}]; mass spectrometric nicotine quantification), the SAEIVS demonstrated excellent linearity of smoke dilution prior to exposure ($R^2 = 0.9951$ for mass spectrometric quantification; $R^2 = 0.9965$ for OD^{400}) and consistent puff-wise exposures across 24 and 96 well plates in cell culture relevant formats (e.g., within inserts). Smoke loss was lower than previously reported for other systems (OD^{400}: 16%; nicotine measurement: 20%). There was good correlation of OD^{400} and nicotine measurements, indicating that OD was a useful surrogate for exposure dosimetry for the product tested. The findings demonstrated that the SAEIVS is a fit-for-purpose exposure system for the reproducible dose-wise exposure assessment of nicotine delivery product aerosols.

KEYWORDS
air-liquid interface, cigarette, exposure characterisation, in vitro exposure, smoke/ aerosol exposure system, whole smoke exposure

1 | INTRODUCTION

In vitro toxicity testing is an important part of the pre-clinical assessment of nicotine delivery products, including combustible cigarettes, heated tobacco products (HTPs) and electronic nicotine delivery systems (ENDS) (e-cigarettes), and can provide an indication of the potential biological effects of the complex chemical mixtures present in smoke/ aerosol generated during use of these products. Smoke/ aerosol extracts have been applied to in vitro cellular systems in various forms. These include addition of smoke fractions (e.g., total particulate matter [TPM], gas vapour phase (GVP) and aqueous smoke extracts) to cell cultures (Czekala, Chapman, et al., 2021; Smart & Phillips, 2021; Thorne et al., 2019) and more recently application of whole smoke to cell cultures directly at the culture air-liquid interface (ALI) (Azzopardi et al., 2015; Li, 2016; Mathis et al., 2013). This perhaps offers the most human-relevant respiratory exposure model, as delivery of whole smoke/ aerosol includes both particulate and GVP compounds, as the cells of the respiratory tract of adult smokers would be exposed to. Such exposure has effectively been applied in in vitro regulatory testing assays (micronucleus test and neutral red uptake [NRU] cytotoxicity assay) (Garcia-Canton et al., 2014; Rudd et al., 2020; Wieczorek et al., 2020) and more recently to 3D human airway epithelial cell cultures (Czekala et al., 2019; Czekala, Wieczorek, et al., 2021).

To achieve whole smoke exposures in a consistent manner for in vitro assessments, products are 'smoked' using smoking robots to defined, standardised puffing regimes. Smoke is then delivered to cells situated within an exposure module (Adamson et al., 2016; Behrsing et al., 2018). There are a number of such systems and set-ups available; these have recently been evaluated by Thorne, Adamson, et al. (2021). Many exposure systems have the functions to dilute the whole smoke/ aerosols generated (with air) to operator-defined puffing regimes, and to smoke multiple products at the same time, to support specific study designs and dose–response testing. A number of in vitro smoke exposure systems have been characterised for their smoke delivery and dilution effectiveness (Scian, Oldham, Kane, et al., 2009; Steiner et al., 2017), and additionally for their ability to generate smoke to defined standardised puffing regimes (Thorne, Wieczorek, et al., 2021). For example, the VitroCell VC-10 S rotary smoking robot is a commonly used, commercially available, exposure system where smoke is carried and diluted via a continuous flow of air through the system, following which cells can be exposed within an exposure module by setting the vacuum rate to draw air from the main air stream into the exposure modules and over the apical surface of the cell layer (Behrsing et al., 2018; Keyser et al., 2019; Thorne & Adamson, 2013). Another commercially available system is the Borgwaldt RM20S smoking machine, which can be adapted with different exposure modules and syringe numbers for delivery of a range of doses (4 up to 8) (Adamson et al., 2011). In the RM20S, the dilution system is not inbuilt into the smoking machine, and tubing lengths are relatively long; therefore, there is a high dead volume present. Finally, there is the Burghart Mimic Smoker-01 (MSB-01), with which smoke generated in five smoking chambers can move through a mixing bag and pump system into an inbuilt cell exposure chamber capable of holding multiwell culture plates (Scian, Oldham, Kane, et al., 2009). The presence of the mixing bag may lead to a relatively high loss of particle matter in the system. Regardless of the various designs available, most ALI exposure setups typically comprise two common elements: the aerosol generation part itself where the initial properties of generated aerosol are determined and an exposure system where the cell cultures in replicate exposure inserts are exposed via ALI to the test aerosol (Zhang et al., 2022).

The aim of any exposure system of the kind described is to deliver freshly generated smoke/ aerosol to cells as rapidly as possible, as longer timeframes taken for smoke/ aerosol to reach the cell cultures can lead to ageing effects in the chemical mixture (Scian, Oldham, Miller, et al., 2009). This may in turn alter the toxicological profile of the aerosol. Greater tubing lengths and smoking machine complexity can also influence the time taken to clean such systems between products/runs. Some machines are adaptable to puff next generation products (NGPs) such as ENDS and HTPs, which can expand the experimental application of the system, and numbers of cigarettes/products smoked at any one time varies (Thorne & Adamson, 2013). Due to the variance between individual product items, multiple replicate smoking runs may be required. Therefore, a more efficient way to achieve this is to generate smoke from several test items at the same time and use the mixture generated as a more representative aerosol for in vitro exposure.

With the aim to address some of these considerations, the custom-built smoke/ aerosol exposure in vitro system (SAEIVS) was developed, the design of which (based on the Burghart MSB-01) allows increased throughput in vitro exposures. The system has the capacity to expose two multiwell cell culture plates (containing cells cultured at the ALI or cell layers aspirated of apical medium for the duration of the exposure) in parallel, in different formats (e.g., different cell culture inserts; in 24 or 96 well plates) dependent on the biological endpoint being tested, at a constant temperature and humidity. Parallel dilution pumps for each chamber allow different smoke dilutions to be delivered to each exposure chamber as required. These are in addition to a main mixing and diluting pump, and fresh filtered, humidified air can be introduced to all of these. The system also contains five smoking chambers with smoking ports that can be adapted to accommodate HTPs and ENDS in addition to combustible cigarettes. Between different products and runs, deep cleaning can be carried out in less than 1 h due to features including easily accessible parts, for example, pumps, and short tubing lengths. The SAEIVS can deliver smoke/ aerosol to cells in under 10 s, which has the advantage of minimising any ageing effects in the chemical mixture. The SAEIVS has been briefly described and utilised to achieve exposures in a number of peer reviewed studies (Czekala et al., 2019; Czekala, Wieczorek, et al., 2021; Rudd et al., 2020; Wieczorek et al., 2020); additionally, it is accredited for its use in smoke/ aerosol testing in the regulatory NRU and micronucleus assays by the Deutsche Akkreditierungsstelle (DAkks), the national accreditation body for the Federal Republic of Germany. The presence of sliding blanking plates, situated above the cell culture plates in each exposure

chamber, can achieve different puff-wise doses across the cell culture plates, and additionally, 'cleaning' puffs of fresh, filtered, humidified air can be programmed into a smoking run. These cleaning puffs mimic smoking behaviour, where intake of fresh air occurs between smoke/ aerosol puffs.

As part of in vitro assessment of smoke/ aerosol exposures, dosimetry is an important step to confirm consistent delivery and provide an indication of relative levels of exposures to the chemical constituents. Measurement of nicotine levels is often used as a marker of exposure and is considered a reliable measure of dosimetry (Adamson et al., 2016; Behrsing et al., 2018). However, this requires equipment and reagents to carry out sensitive mass spectrometric analysis, which in the case of large sample sets may limit the rate of exposure dosimetry assessment. Other methods are employed to quantify smoke delivery, such as the optical density (OD) of a solvent in which smoke has been trapped. This has been applied previously for cigarette smoke dosimetry (Dendo et al., 1998; Phalen et al., 1976; Scian, Oldham, Miller, et al., 2009) and provides a quick indication of its deposition levels. Additionally, it has been demonstrated that the OD of the extract of CF-trapped 3R4F cigarette smoke correlates with the TPM and nicotine concentration measured (https://www.coresta.org/sites/default/files/abstracts/2017_STPOST22_Wieczorek.pdf).

This study aimed to characterise the delivery of fresh whole 1R6F reference cigarette smoke using the SAEIVS in terms of smoke delivery efficiency, smoke distribution across several cell culture formats and the ability to deliver defined levels of exposure. The study used both nicotine levels and OD as dosimetry measurements to assess these and then to compare the outputs of the two methods relative to one another.

2 | MATERIALS AND METHODS

2.1 | Test article

The 1R6F Kentucky Reference Cigarette (University of Kentucky, Lexington, KY) was used in this study. Sticks were conditioned according to International Organization for Standardization (ISO) Guideline 3402 (1999), that is, at least 48 h at 22 ± 1°C and 60 ± 3% relative humidity, prior to smoke generation and smoked according to the ISO 20778 (2018) intense regime (55 mL puff volume, 2 s puff duration, 30 s puff interval, bell-shaped puff profile and ventilation blocking).

2.2 | SAEIVS operation

The SAEIVS (Figure 1) is a five-port smoke/ aerosol generation device specially designed for in vitro exposures. Each cigarette/device holder is positioned in a separate smoking chamber and equipped with its own pump, similar to a linear smoking machine. Selected pumps generate smoke/ aerosol simultaneously and transfer it in sufficient volume to a main mixing and dilution pump. The smoking chambers are arranged in an arc, with each chamber equidistant to the main mixing and diluting pump. Directly adjacent to the main dilution pump, which homogenises the smoke, two further cell exposure chamber-specific pumps are supplied with the mixed smoke via short tubes.

The chamber-specific pumps' function is to transport portions of the aerosol independently of one another to the exposure chamber and, if necessary, to dilute it. Each exposure chamber can therefore be fed with diluted or undiluted aerosol independently of one another. If the influence of GVP alone is to be tested, one of the two chambers can be supplied with the whole smoke/ aerosol and the other with the GVP after passing of the whole smoke/ aerosol through a Cambridge filterpad (CF) inserted between the chamber-specific pump and exposure chamber to trap particulate matter. Because of the different toxicological profiles of different test products and different smoke/ aerosol fractions, the total smoke/ aerosol can be diluted if required with charcoal-filtered and humidified air for delivery to one chamber. However, if required, the GVP can be passed on undiluted into the other chamber.

All pumps can be used for dilution of whole smoke/ aerosol with a maximum diluting factor of 99.9% (smoke concentration = 0.1%). For example, the smoke from 1R6F generated under ISO 20778 (2018) regime used as a standard in the NRU cytotoxicity assay is diluted by maximal percentage of 95% (smoke concentration = 5%), whereas aerosol from HTP and ENDS is usually tested undiluted (based on different cytotoxicity dose profiles).

To minimise smoke/ aerosol ageing, transfer to cells within the exposure chambers is achieved in <10 s, and this is due to the compact construction of the SAEIVS, with the lengths of connection tubes shorter than in many commercial exposure systems. Additionally, the cigarette filter/NGP mouthpiece holders are attached directly to the pumps. These factors enable quick transfer of smoke/ aerosol and minimise the loss of particles in the transfer and diluting processes. It is also of note that specific tubing is used (TYGON 2375; Sigma-Aldrich), which is chemically resistant, plasticiser-free and traps minimal deposits on its surface.

In order to avoid additional dilution of the aerosol due to dead volume in the valves and tubes, these are prefilled with smoke/ aerosol before the test sample is transferred between the pumps. The fast-switching valves ensure that the smoke/ aerosol remains in motion almost continuously during the transfer/dilution process. This also prevents smoke/ aerosol particles from settling in the tubing. Complete transfer of undiluted smoke/ aerosol into an exposure chamber takes ~8 s.

During the exposure phase, each exposure chamber is supplied with the same volume of smoke/ aerosol or GVP regardless of the dilution factor. The volume is equal to the draw volume of a cigarette or device (1 puff). If the aerosol is diluted, the excess volume of aerosol is discarded via an exhaust. Regardless of the volume (e.g., 35 or 55 mL), the puff is blown onto the cells at a constant speed of 78 mL/s. In order to remove the remaining aerosol from the system, it is flushed with 6.6 mL of humidified air immediately after dosing. This corresponds roughly to the dead volume of the tubing from the diluting pump onwards and the distribution manifold.

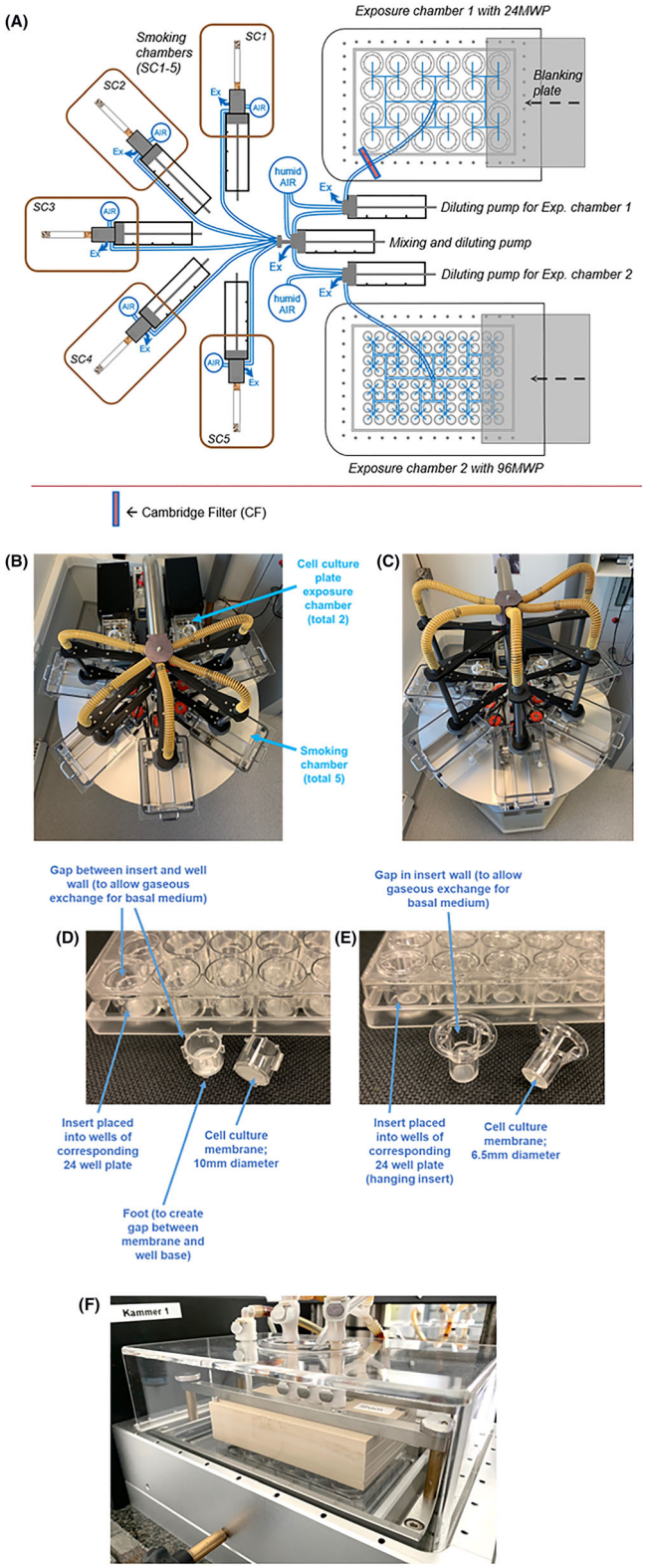

FIGURE 1 (A) Diagram of the smoke/ aerosol exposure in vitro system (SAEIVS), as viewed from above. The system includes five smoking chambers (SCs), which can accommodate cigarettes, electronic nicotine delivery systems and heated tobacco products (in respective runs). Smoke/ aerosol then travels through tubing to a mixing and diluting (with charcoal filtered, humidified air) pump. Each exposure chamber also has a further allocated diluting pump. Exposure chambers can accommodate 24 or 96 well plates, and smoke/ aerosol is delivered to individual wells via ports situated above each. Between the exposure ports and the cell culture plate, a blanking plate can be moved (robotically) to prevent exposure to rows (e.g., to achieve dose-responses). Exhausts (Ex) allow the removal of smoke/ aerosol following each exposure puff. (B and C) Photographic bird's eye images of the SAEIVS. (D and E) Images of the cell culture set-ups used in this study: the 10 mm diameter membrane cell culture inserts (D) are usually used for culture and exposure of V79 cells at the ALI in the micronucleus assay; the 6 mm diameter membrane inserts (E) are usually used for ALI culture and exposure of human lung cell models. (F) Image of the air distribution manifolds present in the cell exposure chambers of the SAEIVS, situated above a 24 well plate containing cell culture inserts as an example (discoloured tubes are the exhaust tubes).

Smoke/ aerosol distributors (specific to 24 or 96 well plate formats) are placed directly above the cell culture plate within the exposure chambers (Figure 1F). The smoke/ aerosol distributor (Scian, Oldham, Kane, et al., 2009) delivers the smoke/ aerosol to each well in a cell culture plate at the same time and in the same volume. A sophisticated distribution system guarantees that the smoke/ aerosol travels the same distance to each above-well port. A single port is aligned to the middle of each well of the multiwell plate; the smoke/ aerosol is blown from above into the wells from a distance of 7 mm. This enables a homogeneous distribution of the smoke/ aerosol to each well. After a dwell time of 3 s, the smoke/ aerosol is rinsed out of the wells with 13 mL (96 MWP) or 20 mL (24 MWP) of air and immediately removed through vacuum air ports also present in the smoke/ aerosol distribution manifold.

Between the aerosol exposure puffs, the plate wells and exposure chambers are additionally cleaned by repeated flushing with 85 mL of air; this also works to mimic smoking-breathing patterns of the adult smoker (Appleton et al., 2015; Jones et al., 2020). As an example, if a puff of smoke is delivered every 30 s, three 'cleaning' puffs of air will be delivered between each smoke puff; if a puff of smoke is delivered every 60 s, seven puffs of clean air will be delivered between each smoke puff. The air used to repeatedly flush and dilute the aerosol is humidified with phosphate buffered saline (PBS) solution to protect the cells from damage. The cells are kept at a constant temperature of 25°C for the duration of the exposure.

In order to evaluate puff/dose-dependent effects, within each exposure chamber, there is a retractable stainless steel (blanking) plate that robotically covers rows of wells after a defined number of puffs. This allows the exposure with smoke/ aerosol to the cells in a puff-dependent manner across the plate.

The SAEIVS can be used to achieve (ALI) exposures in a number of in vitro assays, for example, it is ISO 17025 accredited for use in the micronucleus assay and NRU assay to achieve whole smoke/

TABLE 1 Summary of the key physical parameters of the smoke/ aerosol exposure in vitro system (SAEIVS) for conducting exposure experiments.

Attribute	Details
Temperature of use	25 °C (ambient/room temperature)
Relative humidity	70%–80%
Number of sticks or devices used at the same time	Between 1 and 5
Smoke/ aerosol dilution capability	99.9% (smoke concentration = 0.1%)
Time taken for smoke/ aerosol to reach exposure chamber	8 s

aerosol exposures at the ALI. The NRU cytotoxicity assay has been carried out at the ALI using Beas-2B cells seeded into a Collagen I matrix in 96 well plates (Rudd et al., 2020; Wieczorek et al., 2020). Twenty four well plates have been used in ALI exposures with the SAEIVS, containing 6 mm diameter transwell inserts (as with 3D bronchial epithelial tissue exposures [Czekala et al., 2019; Czekala, Wieczorek, et al., 2021]) or 10 mm diameter inserts (as used in the in vitro micronucleus assay [Chapman et al., 2023; Rudd et al., 2020]). The culture areas for these two insert formats differ by a factor of 2.8. Additionally, the walls of the 6 mm transwell insert contain gaps through which smoke/ aerosol may move, whereas the walls of the 10 mm insert are solid, with space present at the top of the well for air/smoke transfer.

Table 1 outlines the key parameters of the SAEIVS.

2.3 | Nicotine quantification

Nicotine was quantified in samples using liquid chromatography tandem mass spectrometry (LC–MS/MS) (AB Sciex API 6500 QTRAP [SCIEX, USA]). A Gemini NX-C18 column (110 Å, 100 × 2.0 mm, 3 µm) (Phenomenex, USA) was used for the LC (oven temperature 55 ± 1°C), with an injection volume of 5 µL and autosampler temperature of 5°C. The following eluent gradient was applied: 0 min, 2% B (methanol): 98% A (0.05% acetic acid) (flow rate, 400 mL/min); 1.2 min, 65% B: 35% A (400 mL/min); 1.5 min, 95% B: 5% A (400 mL/min); 2.5 min, 98% B: 2% A (400 mL/min); and 3.0 min, 98% B: 2% A (400 mL/min). The following conditions were used for the MS: ion spray voltage, 450 V; ion source temperature, 500°C; multiple reaction monitoring (MRM), 163/132 quantification; and 163/106 qualifier. The limit of quantification (LOQ) for nicotine was 0.25 µg/mL.

2.4 | Optical density measurement

The optical density of TPM in a solvent can be directly measured spectrophotometrically in multiwell plates using absorbance measurements at specific applied wavelengths. In this study, the OD was read at 400 nm (OD^{400}) using the Tecan Sunrise microplate reader. The exposed cell culture area can differ depending on the type of multiwell plate and assay used; therefore, the OD measurement methodology was adapted accordingly. To measure smoke deposition in a 96 well plate, the format used in the NRU assay, TPM was directly captured in 40 µL solvent (PBS) per well. This volume corresponds to the volume filled by cells cultivated on a Collagen I matrix in the NRU assay.

A summary of all experiments used in this study is found in Table 2.

2.5 | Statistical analyses

Statistical analyses were carried out using GraphPad Prism Version 8. The tests carried out for each experiment are outlined in Table 2.

3 | RESULTS

1R6F smoke (combined from five cigarettes) was diluted 50% (smoke concentration = 50%), 80% (20%), 10% (10%), 93.3% (6.66%), 95% (5%) and 97.5% (2.5%), and nicotine concentrations and OD^{400} (Figure 2A and B, respectively) of extracts collected from CFs at the exposure chambers were compared to the pooled levels measured at the smoking chambers. Strong correlations between the dilutions and measurements were observed, using both quantification techniques ($R^2 = 0.9951$ for nicotine quantification and $R^2 = 0.9965$ for OD^{400}).

For 100% smoke delivery, the differences between the measurements at the smoking chambers (of which there are five) and the exposure chambers (of which there are two) were calculated from OD^{400} (n = 6) and nicotine (n = 3) measurements, respectively. Average losses were calculated to be 20.2% (SEM = 2.7) on a nicotine basis and 16.1% (SEM = 1.7) on an OD basis.

The distribution of smoke across both 24 and 96 well plates was assessed. In the 24 well plate format, there was good uniformity of smoke delivered across the wells exposed (columns 2–6), measured on a nicotine concentration in PBS basis, and there were no significant differences between measurements in any of the wells (Figure 3A), or between replicates/the two exposure chambers (Figure 3B). Measurements for the 96 well plate format were taken on both a nicotine concentration in PBS per well and on an OD basis (Figure 4A,B). Both the OD and nicotine measurements correlated strongly (Figure 4C), indicating that the two measurement methods are translatable to one another. Some variability was observed between wells using both analytical approaches, respectively; however, this was not statistically significant. Furthermore, slight variation in delivery (of a set dose) between wells does not appear to translate into different biological responses (outside the normal biological variation) (Chapman et al., 2023; Rudd et al., 2020; Wieczorek et al., 2020).

TABLE 2 Experimental details for the characterisation study.

Experiment	Description	Output	Format	Technical details	Statistical analysis	Figure
1a	Measurement of nicotine concentration in CFs placed at the smoking chamber ports (five CFs) and prior to exposure chambers (two CFs) at different smoke dilutions	Smoke delivery efficiency between the smoking chambers and exposure chambers on a nicotine basis	-	Analyte: CF extracts CF extracts were prepared by incubating the used pads at room temperature in DMSO for 20 min in tubes containing 0.45PP Maxi Spin Filters (Amchro GmbH, Hattersheim, Germany), prior to centrifugation. $n = 3$ Number of cigarettes: 5 per run Puffs/cigarette: 8.6–10 Smoke dilutions: 1/2, 1/5, 1/10, 1/15, 1/20 and 1/40	Simple linear regression analysis	Figure 2A
1b	Measurement of optical density 400 nm (OD^{400}) of extracts from CFs placed at the smoking chamber ports (five CFs) and prior to exposure chambers (two CFs) at different smoke dilutions	Smoke delivery efficiency between the smoking chambers and exposure chambers on an OD^{400} basis	-	Analyte: CF extracts $n = 6$ Number of cigarettes: 5 per run Puffs/cigarette: 8.6–10 Smoke dilutions: 1/2, 1/5, 1/10, 1/15, 1/20 and 1/40	Simple linear regression analysis	Figure 2B
2	Measurement of nicotine concentration in 24 well plate wells exposed to uniform number of puffs of cigarette smoke	Nicotine concentrations across wells and exposure chambers exposed to the same number of puffs of cigarette smoke	24 well plate	Analyte: smoke-exposed PBS (250 µL PBS/well) Number of puffs: ISO 20779 $n = 4$ Undiluted smoke	Two-way ANOVA	Figure 3A,B
3a	Measurement of nicotine concentration in 96 well plate wells exposed to uniform number of puffs of cigarette smoke	As above	96 well plate	Analyte: smoke-exposed PBS (40 µL PBS/well) Number of puffs: ISO 20779 $n = 6$ (288 wells total) After each run with undiluted 1R6F smoke, plates were shaken gently for 2 min; 30 µL from each well was then sampled for nicotine evaluation	Two-way ANOVA Simple linear regression analysis (4c)	Figure 4A,C,D
3b	Measurement of OD in 96 well plates exposed to uniform number of puffs of cigarette smoke	OD^{400} measurements across wells and exposure chambers exposed to same number of puffs of cigarette smoke	96 well plate	Analyte: smoke exposed PBS (40 µL PBS/well) Number of puffs: ISO 20779 $n = 6$ (288 wells total) After each run with undiluted 1R6F smoke, plates were shaken gently for 2 min; OD was then measured directly after this.	Two-way ANOVA	Figure 4B,C

TABLE 2 (Continued)

Experiment	Description	Output	Format	Technical details	Statistical analysis	Figure
4	Measurement of OD in 96 well plates exposed to increasing puffs (across the plates) of cigarette smoke, with different numbers of cigarettes smoked	OD^{400} measurements in wells across plates exposed to increasing puffs of cigarette smoke (across the two exposure chambers). Carried out at different numbers of cigarettes/smoke dilutions to equate to this	96 well plate	Analyte: smoke in exposed PBS (40 µL PBS/well) Puffs/cigarette: 8 Equivalent number of cigarettes in exposure: 0.05, 0.067, 0.1, 0.2, 0.5, 1, 2, 4 and 6 Number of puffs: 2, 4, 6 and 8 $n = 4$	Simple linear regression analysis	Figure 5A,B
5	Measurement of nicotine concentrations within cell culture inserts in 24 well plates and within the medium below to model in vitro exposure set-ups used with the SAEIVS	Concentration of nicotine in extracts from glass discs situated within the cell culture inserts for the exposure duration to increasing puffs of smoke across the plate (both exposure chambers). Concentration of nicotine in the medium situated in the basal medium of the wells	24 well plate with (i) transwell inserts (6 mm membrane diameter) or (ii) cell culture inserts (10 mm diameter)	Analytes: isopropyl alcohol (isopropanol) used to extract smoke deposited on glass plates Volume solvent (isopropanol) used to extract nicotine from glass discs: 0.5 mL for 6 mm discs (in a 0.5 mL Eppendorf tube) and 1 mL for 10 mm discs (in a 1.5 mL Eppendorf tube); shaken at room temperature for 20 min Volume basal medium: Transwell insert (6 mm), 300 µL; cell culture insert (10 mm), 250 µL Puffs/cigarette: ISO 20779 Number of puffs: 6, 12, 18 and 24 $n = 3$	Two-way ANOVA	Figure 6
6	Measurement of nicotine concentrations in the basal medium of 1R6F cigarette smoke exposed cultures in the micronucleus assay (1 dose)	V79 cells grown in 10 mm inserts were exposed at the ALI to 1R6F smoke, and basal medium was extracted for nicotine quantification. The test was replicated in both chambers and on different exposure days.	24 well plate + 10 mm inserts	Puffs/cigarette: ISO 20779 $n = 39$	Descriptive statistical analysis Normal distribution test—D'Agostino and Pearson Unpaired *t*-test (two-tailed) between chambers	Figure 7

Abbreviations: ALI, air-liquid interface; CFs, Cambridge filterpads; OD, optical density; PBS, phosphate buffered saline; SAEIVS, smoke/ aerosol exposure in vitro system.

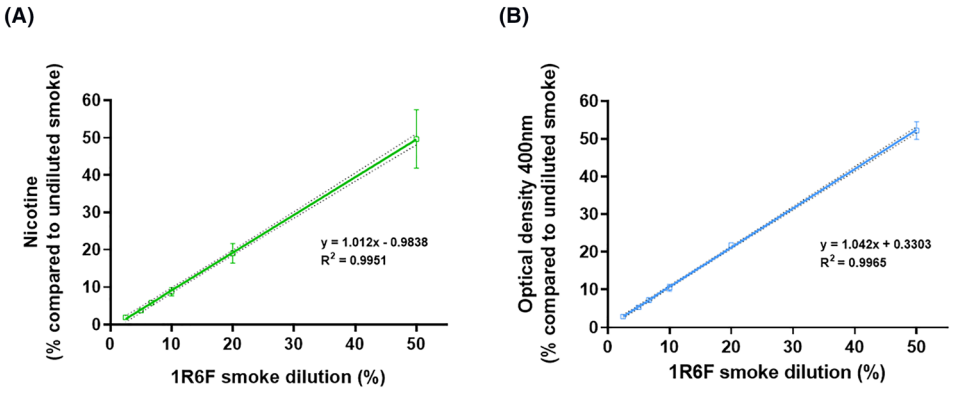

FIGURE 2 Cambridge filterpads (CFs) were placed at the smoking chambers and before the exposure chambers. 1R6F reference cigarettes ($n = 5$ per run) were smoked using the ISO 20778 intense regime, and smoke was diluted by 50% (smoke concentration = 50%), 80% (20%), 10% (10%), 93.3% (6.66%), 95% (5%) and 97.5% (2.5%). Puffs/cigarette: 8.6–10 (according to ISO 20779). Number of replicates (n) = 6 for OD and 3 for nicotine evaluation. Plot (A) shows the ratio of nicotine measured in the CFs correlated with dilution. Plot (B) indicates the ratio of OD measurements (in isopropyl alcohol [isopropanol]) to dilution ($n = 6$). Error bars represent 95% confidence interval.

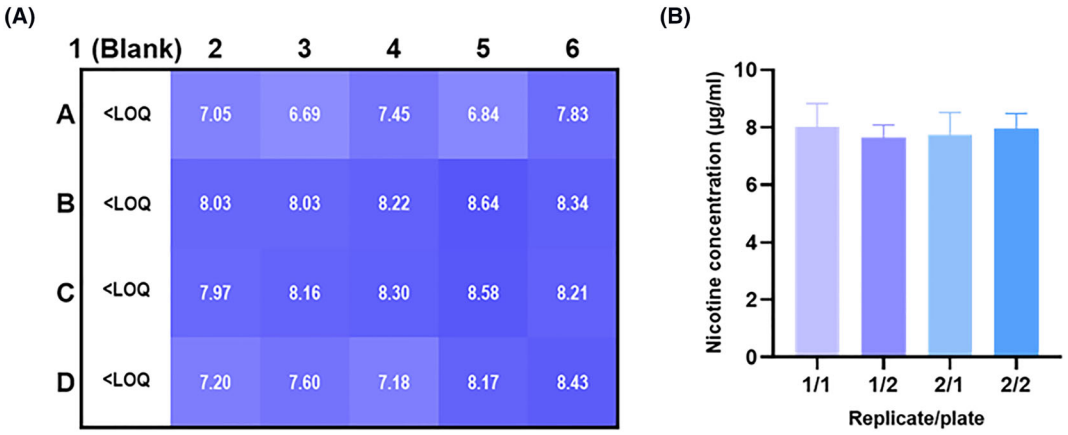

FIGURE 3 (A) Heatmap representation of the distribution of 1R6F smoke (on an average nicotine concentration in phosphate buffered saline (PBS) basis [µg/mL]) across the 24 well plate where columns 2–6 were exposed to the same number of puffs (i.e., according to ISO 20779) and column 1 was blanked with the blanking plate. Panel (B) illustrates the average nicotine concentration measured over the whole (exposed) plates, compared between plates. There were no significant differences between plates (B) or between different wells (A) based on two-way ANOVA with Tukey post-hoc test with 90% confidence interval ($p > 0.01$). ($n = 4$); error bars in panel (B) represent the standard deviation for data in both panels (A) and (B). LOQ, limit of quantification.

Ninety-six well plates were exposed to increasing numbers of cigarettes (and decreasing dilutions of smoke), and the blanking plates were used to expose subsequent columns of wells to increasing numbers of puffs (2, 4, 6 and 8), representing the puff-wise exposures that would be applied during an in vitro exposure. With decreasing exposure to the number of cigarettes, there was a decrease in the range of OD^{400} measurements; however, there were proportional increases in OD measurements with increasing number of puffs for each cigarette number (Figure 5A). The slopes of each puff-wise increase were plotted against number of cigarettes, and this demonstrated strong linearity between the two (Figure 5B).

To further compare the exposures delivered within in vitro testing set-ups, two additional 24 well formats were assessed on a nicotine delivery basis. One is used in the exposure of cells for the in vitro micronucleus assay (OECD 487, 2016), where cells are cultured on 10 mm inserts prior to exposure at the ALI (Chapman et al., 2023; Rudd et al., 2020; Wieczorek et al., 2020). The other is used during studies such as those using 3D human-derived reconstituted lung tissues (Czekala, Wieczorek, et al., 2021), 6 mm membrane diameter transwell inserts. Glass discs were placed onto the apical side of the inserts, exposures were carried out and the glass plates were then extracted with 0.5 mL (6 mm transwells) or 1 mL (10 mm inserts) isopropanol. The subsequently quantified nicotine was proportional to the increasing puff-wise exposures across the plate, and nicotine deposition was proportional to the surface area of the glass plate. However, the total amount of nicotine measured within the basal

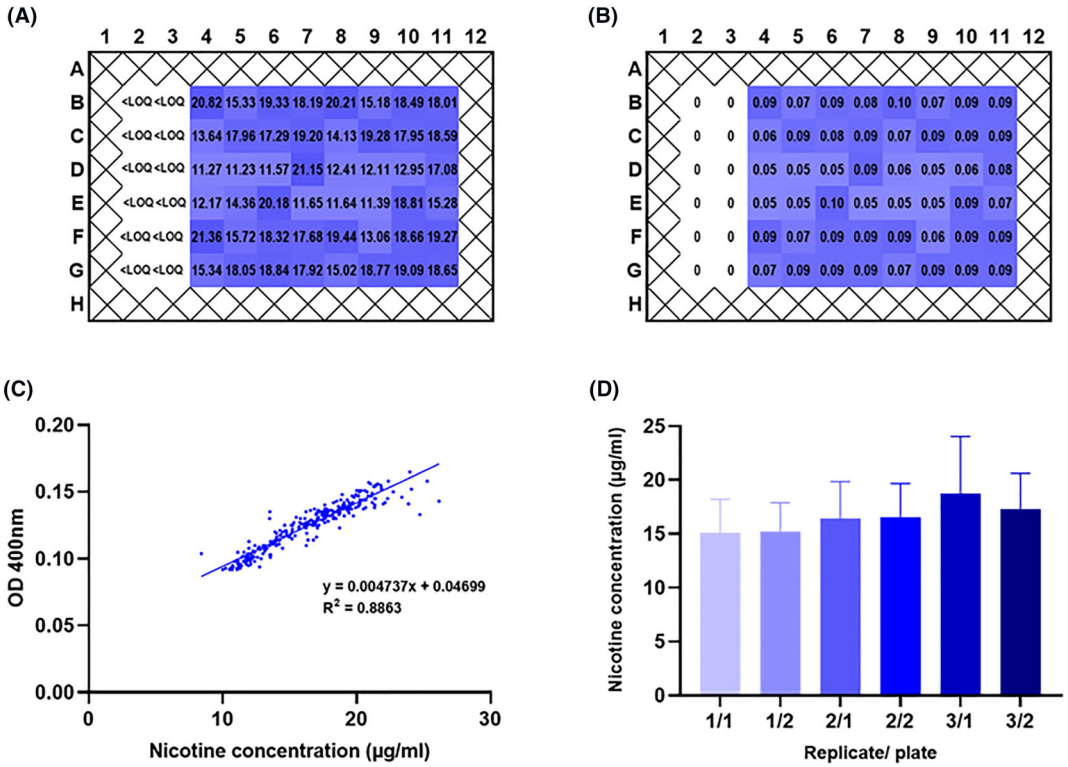

FIGURE 4 Heatmap representations of the distribution of 1R6F smoke, (A) on an average nicotine concentration in phosphate buffered saline (PBS) basis (µg/mL) and (B) an OD^{400} basis (values are background corrected). Columns 4–11 were exposed to the to the same number of puffs, edge wells were not included in the evaluation (in line with the wells exposed in the neutral red uptake biological assay) and columns 2 and 3 were blanked as control (0 dose) wells. Panel (C) illustrates the correlation between nicotine and OD measurements. Panel (D) illustrates the average nicotine concentration measured over the whole (exposed) plates, compared between plates. There were no significant differences between plates based on a two-way ANOVA with Tukey post-hoc test with 95% confidence interval. $n = 6$ (288 wells total); error bars in panel (D) represent the standard deviation for data in panels (A) and (D); individual replicate data points for both nicotine concentration and OD^{400} are represented in panel (C). LOQ, limit of quantification.

medium was significantly higher in the case of the 6 mm transwell set-up, due to the gaps present in the sides of the transwell (Figure 1E).

Nicotine was then measured in the medium of cell cultures exposed to 1R6F smoke for the micronucleus assay (i.e., in 10 mm inserts). Medium nicotine concentrations were found to be consistent between both exposure chambers and exposure days (Figure 7). From the cell free study (Figure 6), it was calculated that the average ratio of mass of nicotine in the basal medium to mass of nicotine deposited on the glass plate was 7.1 ± 1.0 for the 10 mm inserts (54.4 ± 8.6 for 6 mm transwells) and it could therefore be predicted from the basal nicotine measurements in the micronucleus assay that the average mass of 0.013 µg nicotine was deposited onto the cell layer surface during exposure at the dose tested in Figure 7 (ISO 20779).

4 | DISCUSSION

This study aimed to validate the SAEIVS for the repeatability of delivery of whole combustible reference cigarette (1R6F) smoke to in vitro cell culture plates. We demonstrated that the SAEIVS delivered consistent levels of whole smoke to the individual wells of both 24 and 96 well plates and achieved accurate and consistent smoke dilutions (with fresh filtered humidified air) and puff-wise exposures across the plates as required.

4.1 | Smoke delivery efficiency using the SAEIVS

The SAEIVS was firstly tested for the efficiency of smoke delivery from CFs at the smoking ports to CFs situated prior to the exposure chambers. This demonstrated an excellent linearity in the smoke dilution carried out by the SAEIVS and also relatively low smoke losses between these two points (20.2% [SEM = 2.7] on a nicotine basis and 16.1% [SEM = 1.7] on an OD basis). These losses appear to be lower compared to those described by Scian, Oldham, Kane, et al. (2009), who reported the MSB-01 as having 40%–50% losses between the smoking and exposure chambers, with 12%–14% taking place prior to the smoke mixing bag and a further 18%–23% taking place between the smoking bag and exposure chamber smoke distribution manifold. Furthermore, Adamson et al. (2011) reported losses between the smoking ports and point of cell culture exposure for the Borgwaldt RM20S

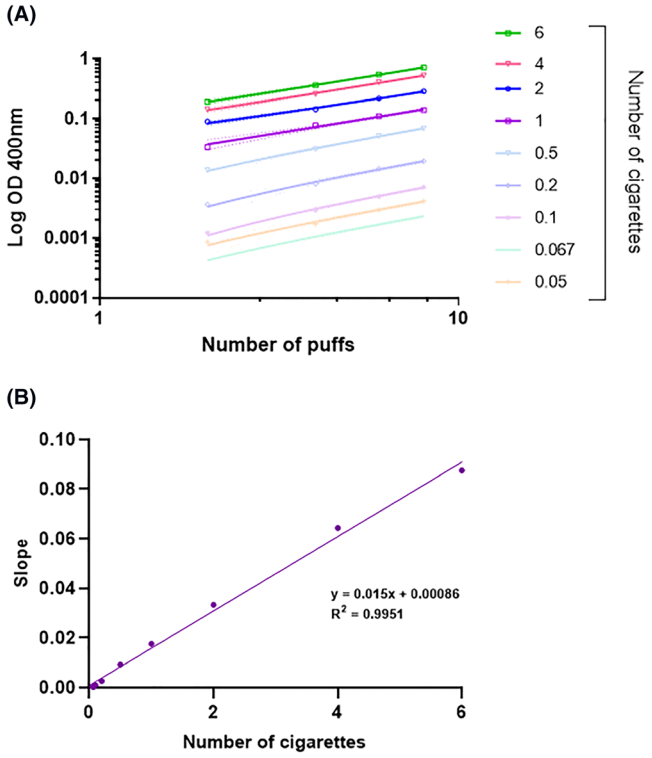

FIGURE 5 (A) OD^{400} measurements in 96 well plates following exposure to increasing puffs (2, 4, 6 and 8) of 1R6F cigarette smoke delivered or diluted to the equivalent of 0.05 to six cigarettes (whole smoke generated to ISO 20779). Data are plotted on a log scale. (B) Plotted slopes of increasing puffs in panel (A) against number of cigarettes in each exposure ($n = 4$).

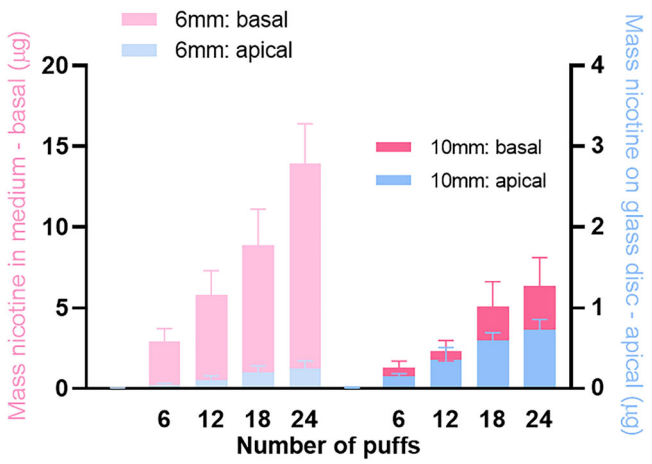

FIGURE 6 Mass of nicotine in cell culture medium below and in the apical compartment (glass plate extracts) of 6 mm transwell cell culture inserts and 10 mm inserts, exposed in a puff dependent manner. Error bars represent standard deviation, $n = 3$ (including four replicate wells per plate [equivalent to 1 column] per number of puffs).

8-syringe smoking machine to be around 47%. More recently, Zhang et al. (2022) reported losses of around 30% for the VitroCell 24/48, however losses of 11% using a modified flow method to reduce the

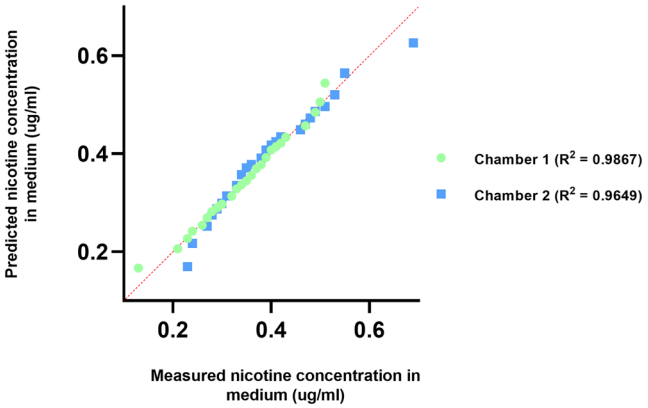

FIGURE 7 Correlation of measured nicotine concentration in the basal medium of V79 cell cultures exposed to 1R6F smoke in the micronucleus assay (x-axis) and the predicted values based on a normal distribution of measured concentrations (y-axis). Values for exposure chambers 1 and 2 are plotted in different colours. $n = 39$.

conductive tubing. The relatively low losses reported in the present study for the SAEIVS may therefore, in part, be attributed to the design feature of short tubing distances in the path between the smoking and exposure chambers, the non-absorbent tubing material and efficiency of the mixing of the smoke in the diluting pumps. Therefore, the pre-study characterisation of the exposure system and the resulting aerosol properties is critical in being able to identify potential further refinements to a system, leading to a better performance for the subsequent biological testing (Scian, Oldham, Kane, et al., 2009).

Here, it was demonstrated that both in 24 and 96 well plates, an even distribution of smoke to each well was achieved during exposure to equal numbers of puffs across the plates (Figures 3A,B and 4A,B,D). Further to this, the use of a blanking plate can achieve puff-wise exposures, and the system's flexibility can be further increased by reversal of the direction of movement of the blanking plate. With this approach, the effects of specific puffs, for example, the first or last puff, can be assessed individually, as opposed to assessing the sum of all puffs delivered to the plate (Wieczorek & Roper, 2010). Outcomes were also replicable between the two smoking chambers (Figures 3B and 4D), further validating this system's ability to deliver controlled, uniform exposures. Smoke delivery to each well is achieved by a distribution manifold with a port situated above each well, which can be changed depending on the plate within the chamber (24 or 96 well). Smoke is therefore delivered to each well in a 'cloud' format, which can achieve a more uniform smoke distribution and deposition compared to, for example, the trumpet delivery system used with the VitroCell VC10S (Oldham et al., 2020); the deposition pattern of particles may be dependent on the trumpet height above the exposure module.

4.2 | Dosimetry methods

OD measurements are commonly used as a measure of smoke/ aerosol capture within a solvent (Dendo et al., 1998; Phalen et al., 1976;

Scian, Oldham, Kane, et al., 2009; Scian, Oldham, Miller, et al., 2009). OD measurement allows high throughput colorimetric analysis of a sample compared to the time required to carry out mass spectrometric analysis of nicotine concentrations within an extract. This study demonstrated that OD^{400} readings for the samples correlated strongly with equivalent measured nicotine levels in 96 well plates (Figure 4C). This indicates that OD measurements can be used as a higher throughput surrogate for dosimetry confirmation for this 96 well cell culture format. This is particularly advantageous for higher throughput multiwell (96) plates as hundreds of samples may require dosimetry for one experiment, which would be costly in terms of time and reagents if put through mass spectrometric analysis. The volume of solute used within the 24 well plates (250 μL PBS/well) did not allow for concentrated enough capture of the smoke within the range of exposures used; however, this lower throughput format would produce a more convenient sample size for mass spectrometry analysis. A limitation of the OD measurement method is that the maximum/optimal absorbance wavelength varies between different products (e.g., between different tobacco blends) and solvents, and this must be optimised prior to measurement. Additionally, for test articles such as NGPs, the aerosols of which demonstrate less complex chemical compositions compared to combustible cigarette smoke (Chapman et al., 2023; Rudd et al., 2020), trapping of sufficient levels of particulate matter for OD analysis may be challenging. The measured OD would also ideally be referenced to a nicotine measurement.

4.3 | Biological relevance

The study was carried out using the 1R6F reference cigarette, which commonly serves as the experimental cigarette comparator in biological studies, including in studies using the SAEIVS (Chapman et al., 2023; Czekala, Wieczorek, et al., 2021; Czekala et al., 2019). Additionally, the SAEIVS has been previously tested with other tobacco blends (i.e., single grade tobacco and reference combustible cigarettes) (Wieczorek et al., 2018), and it has been demonstrated that the exposures achieved in the NRU assay result in statistically distinct dose responses for respective test articles. The system is also extensively used in the testing of NGPs including ENDS and HTPs and again, clear dose responses can be observed in the biological outcomes (Chapman et al., 2023; Czekala et al., 2019; Rudd et al., 2020; Wieczorek et al., 2020). In this study, the dosimetry of 1R6F smoke applied in the in vitro micronucleus assay was assessed using the basal medium below the cell culture transwell inserts. Across exposure days (replicates) and between exposure chambers, smoke delivery, measured by nicotine concentration, was relatively consistent. Based on the ratios of basal and apical nicotine concentrations measured in the system without cells, it was possible to calculate ratios to estimate deposition on the cell layer within the in vitro system using the basal nicotine measurements. However, to confirm this is an effective method of estimation, cell lysates/washes would need to be carried out and analysed for nicotine.

4.4 | Evaluation of the SAEIVS design and study limitations

The SAEIVS has a number of capabilities and advantages, and this study has demonstrated that the system can deliver defined, consistent whole smoke exposures to several cell culture formats. The system can deliver whole smoke/ aerosol to cell cultures in <10 s, and this time includes a dilution step if required. This is in contrast to smoke delivery times reported for other in vitro smoke/ aerosol exposure systems and acts to reduce smoke ageing effects prior to exposure. For example, Scian, Oldham, Miller, et al. (2009) reported changes to the chemical composition of smoke when using the MSB-01. The quick smoke/ aerosol delivery time adds to the increased throughput of the SAEIVS compared to other systems and in fact, the rate limiting factor when carrying out an exposure run is the duration of exposure, which is specific to the experimental design. The presence of the two exposure chambers also increases the throughput of the SAEIVS as they can not only be exposed in parallel according to independent experimental requirements, but can also produce replicate results during experimental repeats. With regards to the processing of biological samples, the SAEIVS offers efficiency as a multiwell plate can be added directly as seeded to the exposure chamber; however, with the VitroCell for example, the exposure module requires an additional preparation step, which would require additional time/resource during a run. The design of the smoking and exposure chambers also enables efficient insertion of cigarettes/NGPs and cell culture plates, respectively.

The system is operated at a temperature of 25°C, selected to align with ambient/room temperature. However, the optimal temperature for cell culture is 37°C, and the functionality to maintain cells at this temperature within the exposure chamber, as achieved with the Borgwaldt RM20S system (Thorne & Adamson, 2013), is not currently present. However, cell cultures are commonly removed from the incubator for maintenance/analyses and are generally resilient to these short periods of environmental change. Furthermore, in vitro models are still limited in how fully they can recapitulate human in vivo conditions, for example, bronchial epithelial cells in situ are not at core body temperature, 37°C, due to the breathing of ambient (cooler) air and the cooling effects both upon inhalation and recondensation of water vapour on the cooler mucosa during exhalation (Haut et al., 2021).

Furthermore, although two dosimetry methods were used in this study, with nicotine measurement the more sensitive of the two, the LOQ for nicotine limited the extent to which dilutions could be assessed, that is, high dilutions may result in a lack of detectable nicotine levels within exposure extracts. Therefore, it is not currently possible to define any linearity of dilutions in this below LOQ range. However, the puff-wise exposures applied within this study were in a range representative of the in vitro exposures carried out using the SAEIVS, and much higher than LOQ; therefore, the data presented here are informative and relevant to this the system's intended use and application.

This study has demonstrated the many advantages the SAEIVS has in the generation and exposure of whole smoke/ aerosol to in vitro models. However, this is currently the only machine of this type; therefore, standardisation across multiple different systems is important to ensure that outcomes can be compared both within and between laboratories. For example, characterisation of systems using a reference product and aligned dosimetry measures have been used for this purpose (Miller-Holt et al., 2022; Thorne, Adamson, et al., 2021). There are working groups who carry out such studies, for example, those of the Institute of In Vitro Sciences (Miller-Holt et al., 2022).

Whilst the focus of this study was to characterise delivery of cigarette smoke using the SAEIVS, the system, as mentioned, can also be used to carry out exposed with other inhaled nicotine products, that is, ENDS and HTPs. To achieve aerosol generation for exposure from such products, the methodology is adapted accordingly. Within the smoking chambers, a multi-functional arm is present, it acts as a lighter for cigarettes; however, its timing and position can be altered, which enables automated button pressing when the system is being used with NGPs such as ENDS and HTPs. Additionally, product-specific holders can be 3D-printed to ensure that the product is positioned at the correct angle and a tight seal is made at any mouthpiece/end to ensure the intended puff is made.

4.5 | Recommendations for in vitro smoke/ aerosol exposure systems

The SAEIVS is one of many in vitro smoke/ aerosol exposure systems (Thorne & Adamson, 2013; Thorne, Adamson, et al., 2021). The experimentation carried out using any such system should therefore be subject to a level of standardisation to ensure reproducible results. This is already in place in terms of ISO smoking regimes, which have demonstrated reproducibility between in vitro set-ups (Thorne, Wieczorek, et al., 2021); however, more standardisation is required with regards to smoke generation equipment. Additionally, pre-conditioning of the system is important to ensure replacement of dead volumes of air, for example, within the distribution manifold and within valves, and therefore continuous flow of smoke/ aerosol. This is an important factor in ensuring the intended smoke/ aerosol delivery level and should be standard practice in the use of such exposure systems.

The system also contains live cell cultures during exposure. In the human lung, a modelling approach by Haut et al. (2021) indicates that the bronchial region is able to condition air to achieve 100% relative humidity, regardless of external air temperature and humidity, with distal lung regions becoming involved in regulation of this only under conditions such as exercise. Therefore, to prevent drying and damage of the cell cultures within the SAEIVS exposure chamber, humidified air is present, and the SAEIVS maintains this within the range of 70%–80% humidity (using PBS). Although 100% humidity is not present, this level has been demonstrated not to affect delicate 3D lung cell models, for example, those used in the study by Czekala, Wieczorek, et al. (2021), whereas application of dry air can damage their cilia (data not shown). Additionally, cultures routinely used in the NRU assay can be exposed for 3 ± 0.5 h without damage to the cell layer.

Whilst the system has been designed for generation and exposure of whole smoke/ aerosol from inhaled nicotine delivery products, the concept and format may be useful for exposures with other products and agents that when used may be inhaled as complex mixtures, for example, fragrances, aerosolised cosmetics and exhaust materials. The dilution system could, for example, be used to generated human exposure-relevant concentrations, with minimal losses between the generation and exposure chamber. However, this would require further product-specific characterisation, and particulate concentration and dosimetry characterisation.

4.6 | Future work

This study has characterised the SAEIVS for in vitro whole smoke exposure testing using the 1R6F reference cigarette. The system has also been used for testing of other combustible tobacco products, but also extensively with ENDS and more recently, HTPs. Within biological test systems, cellular responses to products such as ENDS and combustible cigarettes have been found to be consistent (within the normal biological variation range) between wells (Chapman et al., 2023; Rudd et al., 2020). However, it would be informative to carry out dosimetry studies like the current study using ENDS and HTPs to confirm the aerosol delivery characteristics using the SAEIVS, due to the different compositional nature of their aerosols when compared to combustible cigarette smoke. Additionally, dosimetry of cell layer washes and lysates would be informative to assess how well glass plate deposition and capture can reflect that with ALI cell cultures. This additional data would provide further validation of the SAEIVS for biological assessments. The addition of temperature control functions within the system would also allow cell exposures to be carried out at 37°C.

Whilst the SAEIVS in an important tool in the application of whole smoke/ aerosol exposures in-house, this system is bespoke and therefore external validation is not currently possible, although the system has been part of comparative studies (Thorne, Adamson, et al., 2021). Therefore, this study provides valuable information on the characterisation of the exposure system used in an increasing number of in vitro studies (Chapman et al., 2023; Czekala et al., 2019; Czekala, Wieczorek, et al., 2021; Rudd et al., 2020; Wieczorek et al., 2020).

4.7 | Conclusions

This study has provided dosimetry characterisation of the SAEIVS using reference combustible cigarette, 1R6F over a range of smoke dilutions and puff numbers, and in different cell culture formats. This system allows high throughput exposure of in vitro assessment formats to whole smoke/ aerosol and can be adapted to test different

products, puffing regimes and exposure levels. The consistency in delivery of smoke across wells, dilutions and with different operators demonstrates that the SAEIVS is fit for purpose. The SAEIVS is therefore a valuable part of an in vitro assessment framework, enabling in vitro exposures to all smoke/ aerosol fractions with human cells and therefore increasing the relevance of these biological outcomes to adult smokers.

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CONFLICT OF INTEREST STATEMENT

All authors are employees of Imperial Brands PLC (funders of the work) or its subsidiaries.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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