Genotoxicological assessment of a range of commercially available next generation product aerosols reveals marked reductions in biological activity compared to cigarette smoke

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1. INTRODUCTION

Next generation products (NGPs) offer adult smokers potentially reduced harm alternatives for nicotine delivery. NGPs do not burn tobacco, and therefore produce fewer and significantly lower levels of harmful chemicals compared to cigarette smoke [1, 2]. Distinct categories of NGPs are available, including heated tobacco products (HTPs) and electronic vapour products (EVPs), and within these a range of product designs are available to suit consumer preferences. Whilst the *in vitro* biological effects of such products are being increasingly characterised, a range of testing methods are applied, particularly with regards to the aerosol fractions to which cells are exposed.

2. AIM

This study aimed to compare, on a per puff basis, the biological effects of fresh, whole aerosols from a range of commercially available NGPs (HTPs, EVPs) to 1R6F reference and very low nicotine (VLN) cigarette smoke, using our whole, fresh smoke/ aerosol exposure approach.

3. METHODS

Test articles

Table 1: Summary of products used in the study. EVP = electronic vapour product; HTP = heated tobacco product; VLN = very low nicotine.

Product	Product category	Product details
1R6F	Cigarette	1R6F reference cigarette
VLN King	Cigarette	Very Low Nicotine King cigarette
IQOS 3 Duo + HEETS Russet	HTP	Blade heating technology Rich toasted tobacco stick
IQOS ILUMA + TEREA Russet	HTP	Induction heating technology Rich toasted tobacco stick
Pulze 2.0 + iD Rich Bronze	HTP	Pin heating technology Rich tobacco stick
RELX Classic Tobacco	EVP	Pod-based system (mesh) Classic tobacco e-liquid
ELFA Watermelon	EVP	Pod-based system (mesh) Watermelon e-liquid
Blu 2.0 Golden Tobacco	EVP	Pod-based system (ceramic) Golden tobacco e-liquid

In vitro toxicology assays

Three regulatory *in vitro* toxicological assays were performed:

- ***Neutral red uptake (NRU) assay***: BEAS-2B cells; standard assay protocols were followed in accordance with ISO 17025 [3]. Outcomes were compared on a number of puffs required to induce 20% (EC_{20}) and 50% (EC_{50}) cytotoxicity basis.
- ***Ames test***: *Salmonella* typhimurium strains TA98, TA100, TA102, TA1535, TA1537 (±S9 metabolising system); carried out in compliance with OECD test Guideline 471 [4]. Mutagenic activity was analysed using the slope of the dose-response (fold increase in revertants) using a nonthreshold model and Dunnett's test.
- ***Micronucleus (MN) assay***: V79 cells (±S9); carried out in compliance with OECD test Guideline 487 [5]. Outcomes were assessed for significance using a Chi-Square analysis with Cochran-Armitage trend test. Outcomes were compared for products inducing a positive outcome via EC_{MN3}, the number of puffs required to induce a three-times increase in MN frequency above historic background levels by non-linear regression analysis.

Smoke/ aerosol exposure

For the NRU and MN assays, fresh whole aerosol/ smoke was generated using a bespoke smoking machine, the Smoke/Aerosol Exposure *In Vitro* System (SAEIVS) (Figure 1) to expose cells at the air/liquid interface. The SAEIVS is a five-port smoking machine directly connected to exposure chambers equipped with smoke "distributors" for 24 and 96 well plates. The system is further detailed by Wieczorek *et al.* (2023) [6].

In the case of the Ames assay, whole smoke/aerosol was bubbled through the bacterial cultures, achieved using the Vitrocell VC 10 S-Type Smoking Robot.

The following smoking regimes were applied:

Cigarettes: ISO 20778 [7] (55ml puff volume, 2s puff duration, 30s puff interval, bell shaped puff profile; ventilation blocking)

HTPs: Modified ISO 20778 [7] (55ml puff volume, 2s puff duration, 30s puff interval, bell shaped puff profile; no ventilation blocking). All devices were operated at their highest temperature setting

EVPs: ISO 20768 [8] (55ml puff volume/ 3s puff duration/ 30s puff interval; square shaped puff profile).

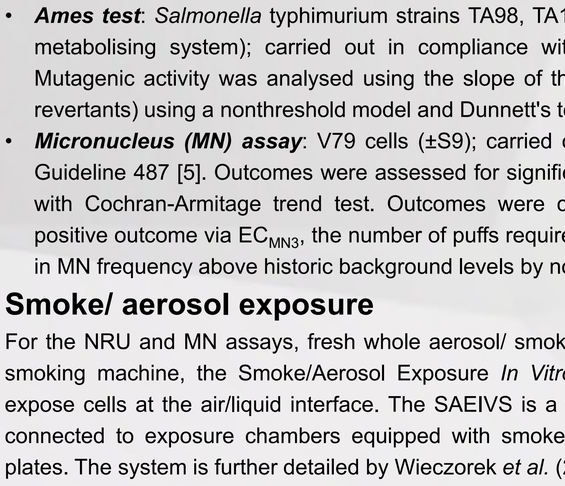

Figure 1: Diagrammatic representation of the Smoke/Aerosol Exposure *In Vitro* System (Wieczorek *et al.*, 2023) [6], consisting of 5 smoking chambers (SCs) and two exposure chambers in which 24 or 96 multiwell plates (MWP) can be exposed.

4.RESULTS

4.1 Cytotoxicity (NRU) outcomes

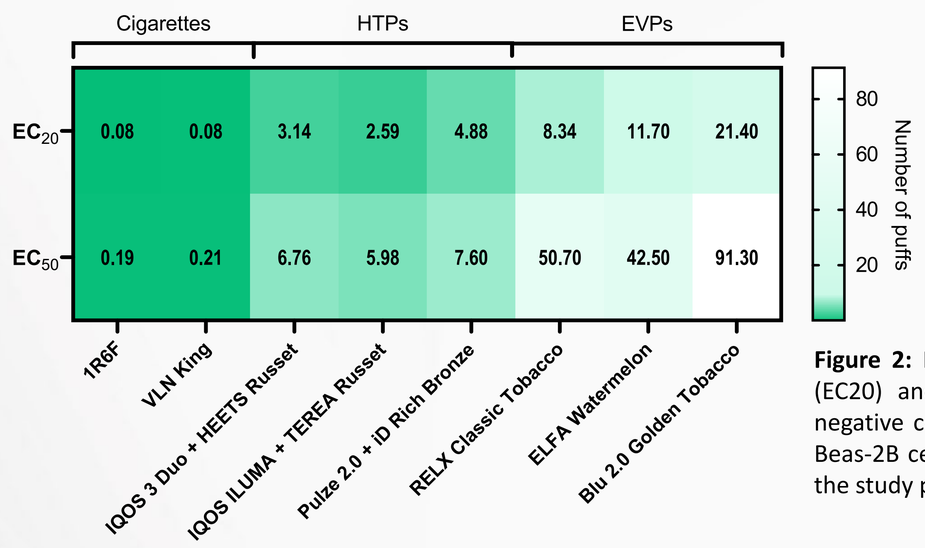

Figure 2: Number of puffs required to induce 20% (EC20) and 50% (EC50) cytotoxicity compared to negative control (puffs of air) following exposure of Beas-2B cells to the fresh whole smoke/ aerosols of the study products.

- Exposure of Beas-2B cells to increasing numbers of puffs of fresh whole smoke/ aerosols revealed distinct cytotoxicity outcomes dependent on product category
- The cigarettes were substantially more potent than the HTPs and EVPs tested, requiring substantially lower numbers of puffs to induce the same effects
- Furthermore, the EVPs were less potent than the HTPs

4.2 Ames test outcomes

- Under the test conditions, the VLN cigarette was classed as mutagenic across the strains tested (+/-S9), as was 1R6F, with the exception of TA1535
- Whilst largely not mutagenic across the strains, the HTPs indicated mutagenic potential in the TA100 strain, however, this was observed at higher numbers of puffs than for the cigarettes
- The fresh whole aerosols of the EVPs did not induce mutagenic outcomes in any of the strains under the test conditions
- Next steps will involve completion of the datasets for all products (i.e., Pulze 2.0 + iD Rich Bronze and Blu 2.0 Golden Tobacco)

Table 2: Classifications in the Ames test for the products included in this study.

Product	TA98 +S9	TA98 –S9	TA100 +S9	TA100 –S9	TA102 +S9	TA102 –S9	TA1535 +S9	TA1535 –S9	TA1537 +S9	TA1537 –S9
1R6F	Mutagenic	Mutagenic	Mutagenic	Mutagenic	Mutagenic	Mutagenic	Not mutagenic	Not mutagenic	Mutagenic	Mutagenic
VLN King	Mutagenic	Mutagenic	Mutagenic	Mutagenic	Mutagenic	Mutagenic	Mutagenic	Mutagenic	Mutagenic	Mutagenic
IQOS 3 Duo + HEETS Russet	Not mutagenic	Not mutagenic	Mutagenic	Mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic
IQOS ILUMA + TEREA Russet	Not mutagenic	Not mutagenic	Mutagenic	Mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic
Pulze 2.0 + iD Rich Bronze	Not mutagenic	Not mutagenic	Mutagenic	Mutagenic	-	-	-	-	-	-
RELX Classic Tobacco	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic
ELFA Watermelon	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic
Blu 2.0 Golden Tobacco	Not mutagenic	Not mutagenic	Not mutagenic	Not mutagenic	-	-	-	-	-	-

4.3 Micronucleus assay outcomes

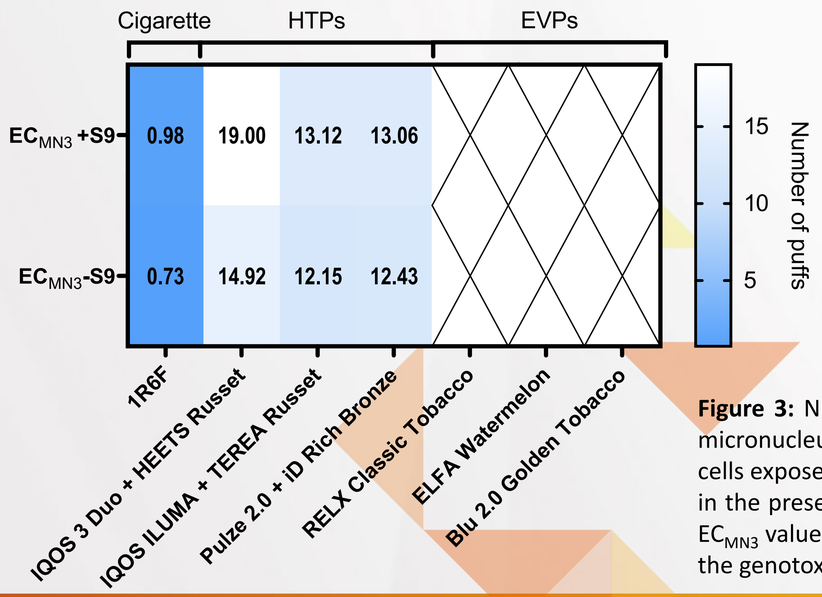

Figure 3: Number of puffs required to induce a three-times increase in micronucleus frequency above historic negative control values in V79 cells exposed to the fresh whole smoke/ aerosols of the products detailed in the presence (+) and absence (-) of S9. For cells marked with an X, no EC_{MN3} value could be derived. Note, the lower the EC_{MN3} value, the higher the genotoxic potential.

- Under the test conditions, 1R6F induced genotoxic outcomes at substantially lower numbers of puffs than compared to the HTPs
- For the EVPs, all outcomes were negative, with the exception of RELX Classic Tobacco, which was classed as equivocal –S9
- A next step will be to assess the VLN King cigarette in the MN assay

5. CONCLUSIONS

- Overall, the cigarettes were consistently the most potent test articles, in terms of their biological activity, across the three assays
- In contrast, responses to the HTPs and EVPs were substantially reduced compared to the cigarettes
- The EVPs were the least potent test articles and did not include positive outcomes in the Ames and MN assays. Where the HTPs induced responses in the Ames test and MN assay, these were at substantially greater numbers of puffs than for the cigarettes
- The results support the proposed placement of nicotine products on a relative risk scale, with cigarette smoking presenting the most risk through exposure to toxicants and NGPs lesser risk, likely due to the presence of fewer and lower levels of toxicants within their aerosols [9]
- Overall, the data suggests that NGPs have the potential to offer harm reduced alternatives to smoking cigarettes and the potential to make a meaningful contribution to tobacco harm reduction
- Future work will involve the testing of further market HTPs and EVPs using the whole smoke/ aerosol exposure approach to add to the weight of evidence for the tobacco harm reduction potential of NGPs

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