In vitro assessment of pod-based e-cigarettes reveals that their aerosol have marked reductions in cytotoxicity, mutagenicity and genotoxicity compared to cigarette smoke

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INTRODUCTION

Fourth-generation, pod-based electronic vapour products (EVPs) are an expanding category of non-combustible nicotine products. They are designed to provide adults smokers who are uninterested in or unwilling to quit smoking with a potentially reduced harm alternative to combustible cigarettes[1]. In previous studies it has been demonstrated that EVPs show 'chemically simple' aerosol when compared to reference cigarettes, and the observed decrease in harmful and potentially harmful constituents (HPHCs) is linked to reduced biological outcomes in CORESTA battery in vitro assays (which are the neutral red uptake (NRU), Ames, and in vitro micronucleus assays)[2]. Due to advancements in e-liquid wicking and atomisation technologies that generate aerosol for inhalation, products with both conventional cotton-based wick atomisers and ceramic based wicks are currently available on the market. Since previous research has been performed on cotton wick atomisers using in vitro assays for cytotoxicity, mutagenicity and genotoxicity, subjecting fourth-generation EVPs to the same tests is a quick and efficient way to compare the potential biological impact of the aerosol of EVPs with different wicking technologies, and cigarette smoke.

Aim

This study aimed to compare the biological effects of the aerosols of three ceramic wick product (blu 2.0™) flavour variants, three cotton wick products (*my*blu™) flavour variants and the 1R6F reference cigarette. E-liquid flavours within the products' pods were matched between the two EVP systems where possible, or the closest match used where the same flavour was not available in both pod systems. Three established biological assays were used: the Ames bacterial reverse mutation, *in vitro* micronucleus (IVM) and Neutral Red Uptake (NRU) assays. The study utilised fresh, whole EVP aerosol and combustible cigarette smoke, to model a more human-relevant exposure scenario, rather than using a single smoke or aerosol fraction, e.g., particulate phase (condensate) or gas-vapor phase only.

METHODS

Test articles

Sample	Type of test article	Wick type	Nicotine strength
1R6F	Reference Cigarette	N/A	N/A
blu 2.0 Tobacco	E-liquid	Ceramic wick	1.6%
blu 2.0 Strong menthol	E-liquid		1.6%
blu 2.0 Blue ice	E-liquid		1.6%
myblu Roasted blend	E-liquid	Cotton wick	1.6%
myblu Strong menthol	E-liquid		1.6%
myblu Blue ice	E-liquid		1.6%

In vitro toxicology

The following regulatory in vitro toxicological assays were performed: Neutral red uptake (NRU) for cytotoxicity in BEAS-2B cells, following standard assay protocols in accordance with ISO 17025; Salmonella typhimurium reverse mutation assay (Ames test) for mutagenicity in TA98 and TA100 in compliance with OECD test Guideline 471[3]; and in vitro micronucleus (IVM) with V79 (±S9) for genotoxicity in compliance with OECD test Guideline 487[4]. Addition of the S9 fraction allows for the metabolism of test articles to be assessed.

Smoke/Aerosol Generation

For the NRU and IVM assays, fresh whole aerosol/smoke was generated using a bespoke smoking machine, the Smoke/Aerosol Exposure In Vitro System (SAEIVS) (Fig. 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.

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.

Smoking regimes used are detailed in Table 1.

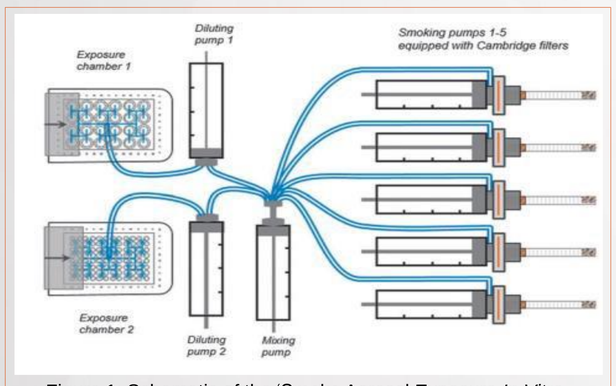

Figure 1: Schematic of the 'Smoke Aerosol Exposure *In Vitro* System' SAEIVS

Table 1: Test product aerosol/smoke was generated using the following regimes.

Sample	Puffing Regime	Puff Volume (ml)	Puff Duration (Seconds)	Puff Interval (Seconds)	Vent Blocking	Puff Profile
1R6F Reference cigarette	ISO 20778	55	2	30	Yes	Bell shaped
EVPs	CRM81	55	3	30	N/A	Square shaped

Data and statistical analysis

Statistical analysis and data handling was conducted in Microsoft Excel and GraphPad Prism. Statistically significant differences between samples were calculated using an analysis of variance (ANOVA) with a post hoc Dunnett's test. Differences were considered statistically significant if the p-value was ≤ 0.05 for the NRU and Ames tests. For the IVM assay, Chi Squared analysis was performed for comparison with negative controls, and Cochran-Armitage trend test to ascertain if there was a dose-response relationship.

RESULTS

Cytotoxicity (Neutral Red Uptake assay)

The NRU assay showed that all products induced dose-dependent increases in cytotoxicity in BEAS-2B cells (Table 2). 1R6F cigarette smoke was observed to be markedly more cytotoxic than all the EVPs tested. The EVP aerosols were between 234-1262 fold less cytotoxic than cigarette smoke on a per-puff basis.

Table 2: EC_{50} values of EVP samples compared to reference cigarette 1R6F.

Sample ID	EC_{50} (puffs)	Fold change compared to 1R6F
myblu Roasted Blend	71.3	353
blu 2.0 Tobacco	109	540
myblu Strong Menthol	47.3	234
blu 2.0 Strong Menthol	188	931
myblu Blue Ice	85.9	425
blu 2.0 Blue ice	255	1262
1R6F Reference cigarette	0.202	N/A

RESULTS CONT.

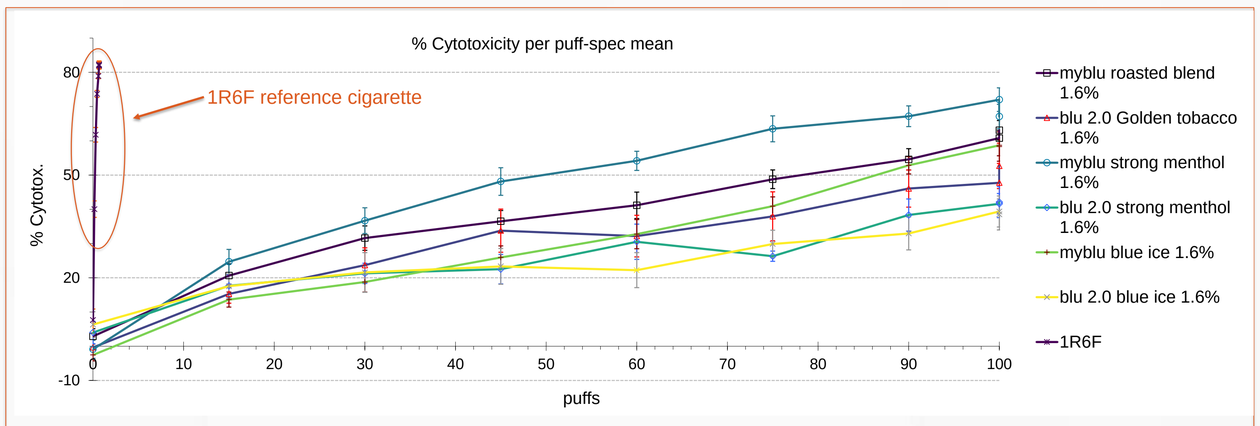

Figure 2: Dose response curves for EVP aerosols and 1R6F cigarette smoke following exposure. 50% cytotoxicity correlates to the EC_{50} in puffs. Error bars = standard error about the mean (SEM)

Overall, all products can be classified as cytotoxic under the test conditions, however EVP products demonstrate marked reductions in cytotoxicity compared to the reference cigarette (figure 2), and ceramic wicks provided a further reduction to cytotoxicity for the flavours tested.

Mutagenicity (Ames assay)

Smoke generated from the 1R6F reference cigarette caused statistically significant and reproducible increases in the number of revertants for both strains +/-S9. In contrast a lack of reproducible, statistically significant increases in revertants was determined for the EVP aerosols, under the conditions of the tests. Maximum puff number for the strong menthol flavours are lower than for the other flavours in both types of wick. This is due to the aerosol of this flavour causing a thinning of the bacterial background colony lawn above the puff numbers in Table 3.

Table 3: Ames test results

Sample ID	S9	Max puff number	Mutagenicity
myblu Roasted blend	+	200	Negative
	-	200	Negative
blu 2.0 Tobacco	+	200	Negative
	-	200	Negative
myblu Strong menthol	+	75	Negative
	-	75	Negative
blu 2.0 Strong menthol	+	125	Negative
	-	125	Negative
myblu Blue ice	+	200	Negative
	-	200	Negative
blu 2.0 Blue ice	+	200	Negative
	-	200	Negative
1R6F Reference cigarette	+	70	Positive
	-	70	Positive

Overall, all EVP aerosols tested negative for mutagenicity. Wicking material did not produce a difference in the outcomes for the EVP flavours tested in this assay. Final classification of mutagenicity is based on defined criteria for the test data, including reproducible induction of a dose response (Table 3).

Genotoxicity (*In Vitro* Micronucleus assay)

Results for the IVM assay shows a clear genotoxic effect for 1R6F cigarette smoke. EVP aerosols were negative for genotoxicity under the conditions of this test due to a lack of reproducible, statistically significant micronuclei being induced.

Figure 3 displays the marked reduction in micronucleus frequencies compared to 1R6F reference cigarette smoke. It also shows a similarity in the biological response of EVPs with cotton and ceramic wicks. This is indicative of the wicks not having any sizeable impact on the genotoxicity of the EVPs.

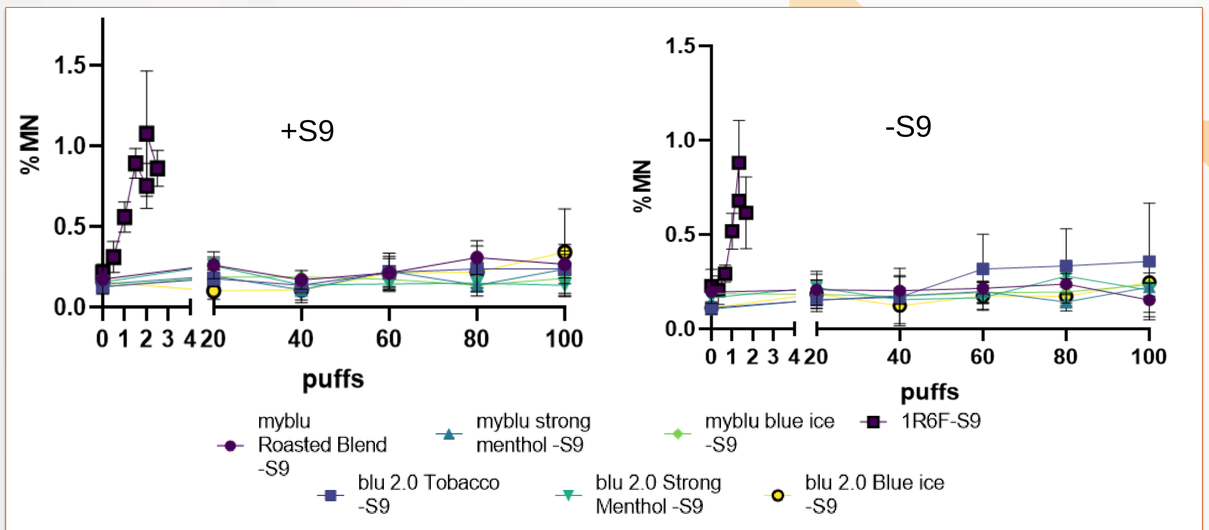

Figure 3: Micronucleus frequencies of EVP aerosols compared to 1R6F cigarette smoke

CONCLUSIONS

-The 1R6F reference cigarette showed clear cytotoxic, mutagenic and genotoxic effects, whereas the EVP products demonstrated marked reductions (ranging from 234 and 1262 fold on average) in activity for cytotoxicity and negative results for mutagenicity and genotoxicity under the conditions of the tests.

-The findings add to the growing body of scientific evidence demonstrating that EVP aerosol is potentially less biologically active in cell systems than cigarette smoke, and that fourth generation EVPs are comparable in biological activity despite the difference in wicking technology used in the atomiser for the e-liquid.

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