Comparative In Vitro Assessment of Heated Tobacco Aerosols to Combustible Cigarettes

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INTRODUCTION

Heated tobacco products (HTPs) are an expanding category of non-combustible tobacco products, which are designed to provide adults smokers with a potentially reduced harm alternative to combustible cigarettes[1]. Due to the evolving regulatory landscape and dynamic nature of innovation of HTPs, new assays are required to quickly determine the potential biological impact of these products pre-clinically.

AIM

In the current study we assessed the aerosol from two HTPs ('Pulze' and another commercially available product, 2 stick variants each from their respective manufacturers, of differing constructions and flavours) and compared the biological response to 1R6F reference cigarette smoke in the Ames, *In Vitro* Micronucleus (IVM) and Neutral Red Uptake (NRU) assays. In the present study we utilised fresh, whole HTP aerosol and combustible cigarette smoke, to represent a human-relevant exposure, rather than using a single smoke / aerosol fraction, e.g., particulate phase (condensate) or gas-vapor phase only.

METHODS

Test Articles

- 1R6F Reference Cigarette (University of Kentucky)
- Commercially available Heated Tobacco Product (cbHTP) with ceramic heater blade technology heats to a maximum of 350ºC, two stick varieties: Rich Tobacco (RT) and Toasted Tobacco (TT) aromas
- Commercially available Heated Tobacco Product, "Pulze" (cpHTP) with ceramic heater rod technology heats to a maximum 345ºC, two "iD stick" varieties: Balanced Tobacco (BT) and Menthol (MT) aromas

***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; and in vitro micronucleus (IVM) with V79 (±S9) for genotoxicity in compliance with OECD test Guideline 487. Cells were exposed to smoke or aerosol at the air liquid interface using the internal smoking machine 'smoke aerosol exposure in vitro system' (SAEIVS) (Burghart Tabaktechnik, Wedel, Germany) for NRU and IVM and using the smoking machine Vitrocell VC10S (VITROCELL Systems GmbH, Waldkirch, Germany) for the Ames assay.

Smoke/Aerosol Generation

For the NRU and IVM assays, fresh aerosol/whole smoke was generated using a bespoke smoking machine SAEIVS (Fig. 1). The SAEIVS is a five-port smoking machine directly connected with the exposure device and equipped with smoke "distributors" for 24 and 96 multiwell 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.

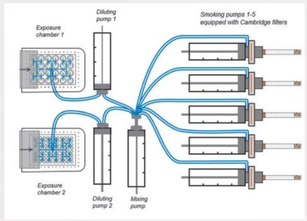


Figure 1: Schematic of the 'Smoke Aerosol Exposure In Vitro System' SAEIVS

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

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
HTPs	Modified ISO 20778	55	2	30	N/A	Bell shaped

Data and statistical analysis

All data and statistical analysis were conducted using Microsoft Excel and GraphPad Prism. Statistically significant differences between samples were calculated using ANOVA with posthoc Dunnett's test. All differences were considered statistically significant with a p-value ≤ 0.05.

RESULTS

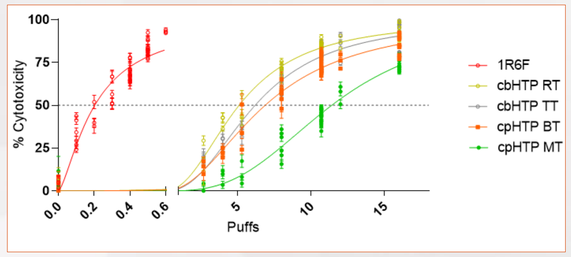
The Neutral Red Uptake assay shows marked reductions in HTP aerosol cytotoxicity compared to reference cigarette smoke

The results are reported as the EC_{20} and EC_{50} values with corresponding confidence intervals and compared to smoke from 1R6F reference cigarette. Fresh smoke from 1R6F reference cigarette and aerosol from HTP induced >50% cytotoxicity to BEAS-2B cells in dose dependent manner and all products are considered cytotoxic under the conditions applied (See Table 2).

Table 2: EC_{20} and EC_{50} values for each test article

Sample ID	EC_{50} (puffs)	95% Confidence interval From	95% Confidence interval To	EC_{20} (puffs)	95% Confidence interval From	95% Confidence interval To
cbHTP: RT	5.11	4.95	5.27	2.73	2.57	2.88
cbHTP: TT	6.2	5.99	6.41	3.46	3.24	3.69
cpHTP: BT	6.88	6.67	7.09	3.58	3.35	3.81
cpHTP: MT	11.4	11.2	11.60	7.15	6.89	7.41
1R6F Reference Cigarette	0.203	0.191	0.215	0.077	0.067	0.087

Figure 2: Dose response curves for the different test aerosols and reference cigarette smoke following exposure. The grey dotted line indicates 50% cytotoxicity. Error bars = SEM



All HTP aerosols demonstrated marked cytotoxicity reductions compared to cigarette smoke on a per puff basis (See Table 3):

Sample ID	EC_{50} (puffs)	Fold induction compared to 1R6F
cbHTP: RT	5.11	25
cbHTP: TT	6.2	31
cpHTP: BT	6.88	34
cpHTP: MT	11.4	56
1R6F Reference Cigarette	0.203	1

The Ames assay shows that HTP aerosol is not mutagenic

Smoke generated from the 1R6F reference cigarette caused a statistically significant and reproducible increase in the number of revertants ±S9 mix. Fresh aerosol from the cpHTP BT variant showed evidence of causing a dose dependent and statistically significant increase in the number of revertants in TA100-S9, but this effect was not reproducible and was classified as equivocal under the conditions applied in this study. Fresh aerosol from all other products were not mutagenic under the conditions applied in this study.

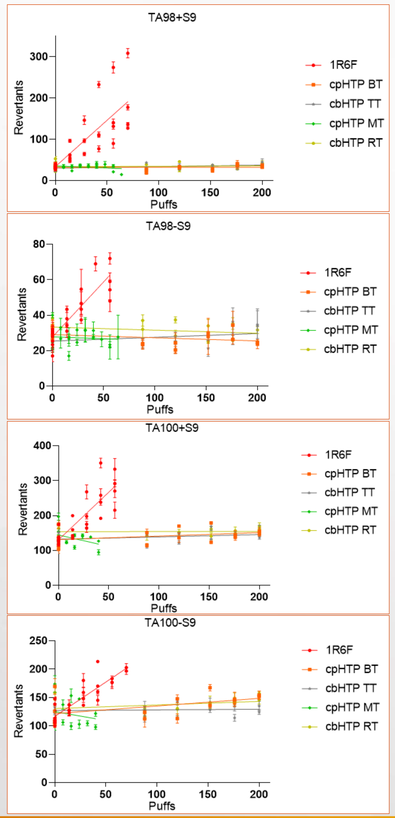


Figure 3: Dose response of smoke and different aerosols in Ames test with *S. Typhimurium* TA98 +S9.

Figure 4: Dose response of smoke and different aerosols in Ames test with *S. Typhimurium* TA98 -S9.

Figure 5: Dose response of smoke and different aerosols in Ames test with *S. Typhimurium* TA100 + S9.

Figure 6: Dose response of smoke and different aerosols in Ames test with *S. Typhimurium* TA100 -S9.

***In Vitro* Micronucleus assay shows marked reductions in HTP aerosol genotoxicity compared to reference cigarette smoke**

Statistically significant, dose-dependent and reproducible increases in relative micronucleus frequencies as compared to the negative control cultures were induced by smoke from cigarette and HTP aerosols (within acceptable toxicity levels). The HTP aerosols and the cigarette smoke were classified as genotoxic.

The data indicate that aerosol from the different HTP sticks were 10.1 to 15.4 fold less genotoxic in the presence and 10.7 to 17.1-fold less genotoxic in the absence of a metabolic activating system, compared to combustible cigarette smoke.

Figure 7: Dose response curves of smoke and different test aerosol in the IVM assay in the presence of S9. The black dotted line indicates the 3-fold increase corrected by background micronucleus frequencies.

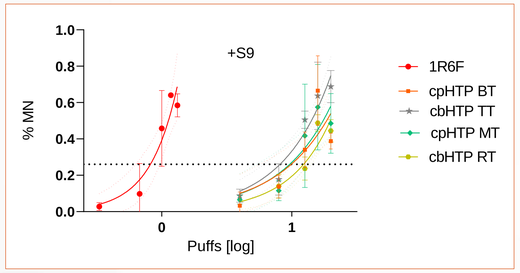
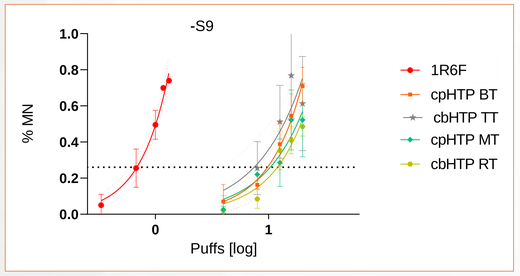


Figure 8: Dose response curves of smoke and different test aerosol in the IVM assay in the absence of S9. The black dotted line indicates the 3-fold increase corrected by background micronucleus frequencies.



CONCLUSION

- The 1R6F Reference cigarette showed clear cytotoxic, mutagenic and genotoxic effects, whereas the HTP products had marked reductions (10-56 fold) in activity for these endpoints under the conditions of the tests.
- The findings add to the growing body of scientific evidence that HTPs have a reduced emission profile with fewer and substantially lower HPHCs, shown here to directly translate into a potentially less harmful risk profile compared to continued combustible tobacco smoking.
- However, the results presented here need to be further substantiated in studies utilising advanced *in vitro* techniques and clinical assessments.

REFERENCES

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