Comparative Toxicological Assessment of Heated Tobacco Product Aerosol Versus Combustible Cigarette Smoke Using In Vitro Regulatory Cytotoxicity and Genotoxicity Assays

DJ Smart[1], U Doshi[2] and SJ Harbo[1]

[1] JT International SA, Rue Kazem-Radjavi 8, CH-1202 Geneva, Switzerland; [2] Altria Client Services LLC, Richmond, VA, United States

INTRODUCTION

- The Ploom® system (hereafter *Ploom*) is a heated tobacco product (HTP) consisting of heated tobacco sticks (HTS) and a battery-powered tobacco heating device
- *Ploom* underwent comprehensive testing to assess its reduced risk potential relative to combustible cigarettes.
- *Ploom* mainstream emissions characterization data recently demonstrated on average > 90% reduction in harmful and potentially harmful constituents vs. the reference cigarette, 1R6F (Jackson et al., 2024).
- As part of continued nonclinical testing, *Ploom*-derived aerosol fractions were assessed for cytotoxic and genotoxic potential in a 21 CFR Part 58-compliant in vitro study comprised of the neutral red uptake (NRU) cytotoxicity assay, bacterial reverse mutation (Ames) assay and non-cytokinesis block in vitro micronucleus (ivMN) genotoxicity assay.
- 1R6F reference combustible cigarette-derived smoke fractions were tested alongside *Ploom* aerosol fractions in each in vitro assay to provide a combustible cigarette context.

MATERIALS & METHODS

- **Control & Test Articles:** Reference combustible cigarette (1R6F); Tobacco heating device and 4 heated tobacco sticks (HTS) varieties (menthol: MX5 and MX3; tobacco: RX4 and R8)
- **Generation & Preparation of Smoke/Aerosol Fractions:**

1R6F Cigarette Smoke Fractions: Total particulate matter (TPM) and gas-vapor phase (GVP) were collected and prepared according to the ISO 20778 regime. In brief, a rotary smoking machine (RM20) was used to smoke cigarettes, and the TPM was collected onto a 44 mm Cambridge filter pad (CFP) and extracted in dimethyl sulfoxide (DMSO), while the GVP was collected by bubbling the filtered cigarette smoke into an ice-cooled glass impinger containing calcium-magnesium-free phosphate-buffered saline (CMF-PBS) (Table 1).

Ploom Aerosol Fractions: Aerosol collected matter (ACM) and GVP were collected and prepared according to the modified ISO 20778 regime (no ventilation hole blocking). Briefly, aerosols from HTS via the device were generated using a rotary smoking machine (RM20D), and the ACM was collected onto a 44 mm CFP and extracted in DMSO, while the GVP was bubbled into an ice-cooled glass impinger containing CMF-PBS (Table 1).

Smoke/Aerosol Fraction Chemical Characterization: The TPM/ACM fraction was analyzed for nicotine, glycerol, and menthol, while GVP was analyzed for select carbonyls (acetaldehyde, acrolein, crotonaldehyde, and formaldehyde) using validated analytical methods (Figure 5).

Table 1. Smoke/aerosol fraction generation & collection information.

Product Category	Name	Number of Article Accumulations Per Collection	Solvent Volume (mL)	
			DMSO (for TPM/ACM)	CMF-PBS (for GVP)
Combustible Cigarette	1R6F	13 or 20	20, 26 or 85	15
HTP	MX5	100	15	20
	MX3			
	RX4			
	R8			

- **In Vitro Test Battery:**

Testing Approach: Three independent replicates of each in vitro assay were carried out.

NRU Cytotoxicity Assay: The cytotoxicity assay was performed according to Health Canada official test method T-502, using CHO-WBL cells. Maximum concentrations tested were either based on 1% v/v (TPM/ACM) and 10% v/v (GVP) or were limited by cytotoxicity after 24±0.5 hours of exposure. Results are normalized to TPM/ACM or TPM/ACM equivalent and reported as % relative viability to vehicle control. The theoretical concentration inducing 50% cytotoxicity (IC_{50}) was calculated from concentration-response curves using a four-parameter logistic mathematical model in GraphPad Prism statistical software V9.0.4. Concentration-cytotoxicity data and non-linear transformation plots are shown in Figure 1, cytotoxicity of *Ploom* relative to 1R6F is shown in Figure 2 and IC_{50} values are shown in Table 2.

Ames Mutagenicity Assay: The mutagenicity assay was conducted according to OECD 471, using 5 bacterial (*Salmonella typhimurium*) strains: TA98, TA100, TA102, TA1535 and TA1537; each tested with and without metabolic activation (S9). Maximum volumes tested were 50 µL/plate (TPM/ACM) and 100 µL/plate (GVP). Results from the TA98+S9 condition show the average number of observed revertants against TPM/ACM concentrations per plate (Figure 3).

ivMN Genotoxicity Assay: The genotoxicity assay was conducted according to OECD 487, using CHO-WBL cells, under 3 treatment schedules (short-term±S9 and long-term-S9). Maximum concentrations tested were based on 1% v/v (TPM/ACM) and 10% v/v (GVP), and cytotoxicity was assessed using the relative increase in cell count (RICC) index. The highest exposure concentration selected for MN scoring was either the concentration producing 50-60% cytotoxicity or the highest concentration of the fraction (if cytotoxicity was <50%). %MN responses (analyzed via microscopy) are shown for TPM/ACM in the short-term treatment schedule +S9 (Figure. 4).

RESULTS: NRU CYTOTOXICITY ASSAY

Figure 1. Cytotoxicity curves of 1R6F combustible cigarette- and *Ploom*-derived TPM/ACM (A) and GVP (B), respectively.

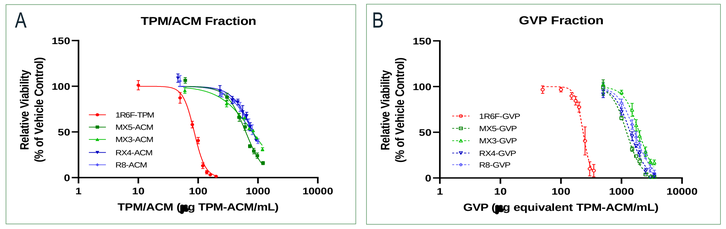


Figure 2. Relative cytotoxicity of *Ploom* compared to 1R6F combustible cigarette.

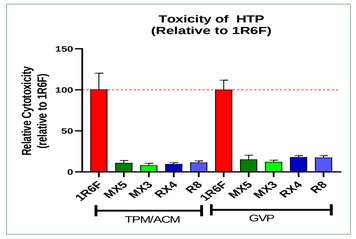


Table 2. IC_{50} potency values of smoke/aerosol fractions.

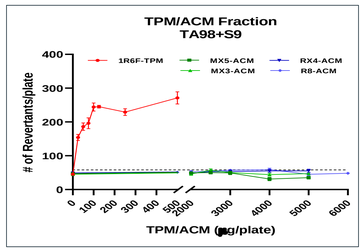
Control or Test Article	IC_{50} (µg TPM or ACM/mL) Mean ± SD	
	TPM or ACM	GVP
1R6F	90.3 ± 18.7	258 ± 32
MX5	856 ± 208	1846 ± 622
MX3	1197 ± 352	2178 ± 455
RX4	964 ± 212	1446 ± 163
R8	1001 ± 193	1492 ± 202

Summary of findings:

- 1R6F-derived TPM and GVP induced cytotoxicity in a reproducible, concentration-dependent manner.
- ACM and GVP derived from all *Ploom* HTS varieties also induced reproducible, concentration-dependent cytotoxicity.
- However, the cytotoxic potency of *Ploom* aerosol fractions was markedly lower (6-13-fold) than that of 1R6F smoke fractions.
- This cytotoxicity profile of *Ploom* aerosol fractions in the NRU cytotoxicity assay is consistent with that of other HTPs reported in the scientific literature despite being assessed in a different mammalian cell line (Schaller et al., 2016 and Thorne et al., 2018).

RESULTS: AMES MUTAGENICITY ASSAY

Figure 3. Mutagenicity of TPM/ACM fractions derived from 1R6F combustible cigarette and *Ploom* in strain TA98 (+S9).

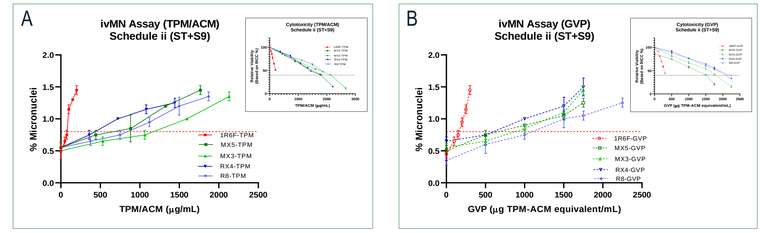


Summary of findings:

- 1R6F TPM was mutagenic, demonstrating concentration-dependent increases in revertant colonies in strain TA98 (+S9) across all 3 replicates.
- Increases in revertant colonies were also observed for strains TA100 (+S9), TA1537 (+S9) and TA100 (-S9) in response to 1R6F TPM. However, these responses did not meet all the evaluation criteria across each replicate and, thus, were concluded as non-mutagenic overall.
- ACM from all *Ploom* HTS varieties were non-mutagenic in all five bacterial strains (±S9) when tested to the limit of cytotoxicity or maximum feasible dose (up to 10-12-fold more concentrated relative to 1R6F TPM; up to 5-6 mg ACM/plate). Overall, the response from all *Ploom* HTS were comparable.
- GVP derived from both 1R6F and the *Ploom* HTS varieties were non-mutagenic under the test conditions (data not shown).
- The non-mutagenic profile of *Ploom* aerosol fractions in the Ames assay is analogous to that of other HTPs reported in the scientific literature (Schaller et al., 2016 and Thorne et al., 2018).

RESULTS: ivMN GENOTOXICITY ASSAY

Figure 4. Genotoxicity of TPM/ACM (A) and GVP (B) fractions along with respective cytotoxicity curves (inset) of 1R6F combustible cigarette and *Ploom* from treatment schedule ii (ST+S9).

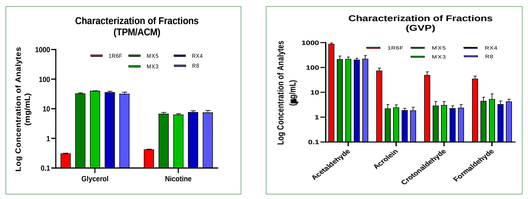


Summary of findings:

- 1R6F-derived TPM and GVP induced reproducible genotoxic responses in the 3 treatment schedules.
- Similarly, *Ploom*-derived ACM and GVP also induced reproducible genotoxic responses in the 3 treatment schedules.
- However, the genotoxic potency of *Ploom* fractions was overall markedly lower (6-44-fold) than that of 1R6F smoke fractions.
- This genotoxicity profile of *Ploom* aerosol fractions in the ivMN genotoxicity assay is consistent with that of other HTPs reported in the scientific literature despite some differences in the test methodologies used (Schaller et al., 2016).

RESULTS: SMOKE/AEROSOL FRACTION CHEMICAL CHARACTERIZATION

Figure 5. Representative TPM/ACM and GVP chemical characterization data (from Ames assay).



TPM/ACM stock [mg TPM-ACM/mL]: 10 (1R6F), 245 (MX5), 245 (MX3), 200 (RX4), 200 (R8)
GVP stock [mg TPM-ACM equivalent/mL]: 57 (1R6F), 178 (MX5), 180 (MX3), 155 (RX4), 152 (R8)

Summary of findings:

- In terms of carbonyls, 1R6F-derived GVP was ≥4.5-fold more concentrated than *Ploom*-derived GVP.
- In contrast, *Ploom*-derived ACM was ≥54-fold more concentrated than 1R6F-derived TPM in terms of glycerol, menthol (data not shown) and nicotine.

CONCLUSIONS

NRU Cytotoxicity Assay: *Ploom*-derived aerosol fractions (both ACM and GVP) were markedly less potent (6-13-fold) in terms of cytotoxicity than the smoke fractions derived from the 1R6F reference cigarette.

Ames Mutagenicity Assay: *Ploom*-derived aerosol fractions (both ACM and GVP) were non-mutagenic when assessed up to the limit of cytotoxicity or highest feasible dose. The 1R6F-derived TPM, in contrast, was unequivocally mutagenic, while its GVP was non-mutagenic under the test conditions.

ivMN Genotoxicity Assay: *Ploom*-derived aerosol fractions (both ACM and GVP) were genotoxic in all three treatment schedules, as were 1R6F-derived TPM and GVP. However, the genotoxicity potency was approximately an order of magnitude lower for the *Ploom* fractions versus the 1R6F fractions.

Final Conclusions:

- ***Ploom*-derived aerosol fractions exhibited overall lower in vitro cytotoxic, mutagenic and genotoxic potential compared to the smoke fractions derived from the 1R6F reference cigarette.**
- **Our results biologically corroborate the *Ploom* mainstream emissions characterization data which demonstrate an overall 90% reduction in harmful and potentially harmful constituents vs. 1R6F (Jackson et al., 2024).**
- ***Ploom*'s in vitro toxicological profile is similar to that of HTPs reported in the scientific literature.**

REFERENCES

- Jackson TJ et al., 2024. Product Science/Product Technology Groups, CORESTA Congress, Edinburgh. ST45
- Schaller JP et al., 2016. Evaluation of the Tobacco Heating System 2.2. Part 2: Chemical composition, genotoxicity, cytotoxicity, and physical properties of the aerosol. Regul. Toxicol. Pharmacol. 81 Suppl. 2: S27-S47.
- Thorne D et al., 2018. Assessment of novel tobacco heating product THP1.0. Part 7: Comparative in vitro toxicological evaluation. Regul. Toxicol. Pharmacol. 93: 71-83.