Comparative *In Vitro* Toxicological Assessment of Electronic Nicotine Delivery Systems (ENDS)

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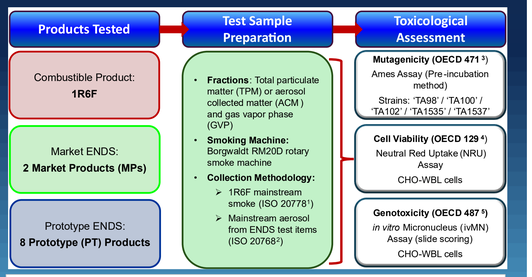
Background and Purpose

Electronic nicotine delivery systems (ENDS) are an expanding category of inhalable, non-combustible tobacco products that have generated significant scientific and regulatory interest due to their potential role in tobacco harm reduction for adult smokers. Since their introduction, ENDS have evolved substantially in design and functionality, encompassing a wide range of product formats, including disposable devices, rechargeable cartridge-based systems, and refillable tank or "mod" systems. Furthermore, ENDS are available in a wide variety of formulations that differ in nicotine concentration, solvent composition (e.g., propylene glycol and vegetable glycerin ratios), and flavorings, contributing to substantial variability in aerosol characteristics and user exposure. Given the diversity of ENDS devices and formulations, *in vitro* toxicology assays play a critical role in the early evaluation of ENDS prototypes by enabling comparative assessments of aerosol-induced biological responses and the identification of a potential hazard. The current study evaluated the toxicity profile of 8 prototype and 2 commercially available ENDS products against a combustible test item (1R6F) using established regulatory *in vitro* toxicity assays.

Materials and Methods

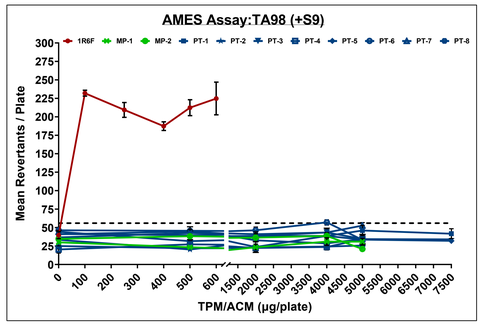
IN VITRO TOXICOLOGICAL ASSESSMENT WORKFLOW



- TPM test samples were collected at concentrations up to 40 mg TPM/mL in DMSO and GVP test samples at concentrations up to 40 mg TPM equivalent/mL in PBS.
- ACM test samples were collected at concentrations of 200 mg ACM/mL in DMSO and GVP test samples at concentrations of 200 mg ACM equivalent/mL in PBS.
- The maximum test sample concentration evaluated in the *in vitro* assays was either limited by toxicity or by solvent restrictions.
- For all test items, chemical analyses including the measurement of nicotine, glycerol (VG), propylene glycol (PG), and water were performed on the TPM and ACM test samples while the content of select carbonyls was determined from GVP test samples.

Results

Figure 1. Ames Assay. TPM/ACM and GVP test samples were tested at concentrations up to 1000 µg/plate (1R6F), and up to 10000 µg/plate (ENDS). Representative data are shown for TPM/ACM test samples in strain TA98 with (+S9) metabolic activation. Data are normalized to mass.

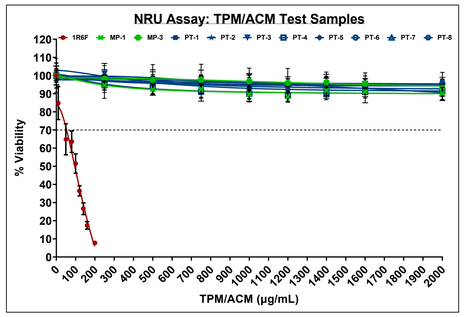


Mutagenicity

- 1R6F induced mutagenicity in strains TA98 (+/-S9) & TA1537 (+S9).
- Tested ENDS products were negative in all tested strains even at 10-15-fold higher concentrations than 1R6F.
  - One to two top concentrations from ENDS products induced toxicity (data not shown), suggesting that the selected top dose of 10 mg/plate was sufficient for the Ames assay.
- All GVP fractions were negative.

GVP data are not shown.

Figure 2. Neutral Red Uptake (NRU) Assay. TPM/ACM and GVP test samples were tested at concentrations up to 250 µg/mL (1R6F), and up to 2000 µg/mL (ENDS). Representative cytotoxicity results are shown for the TPM/ACM test samples. Data are normalized to mass.

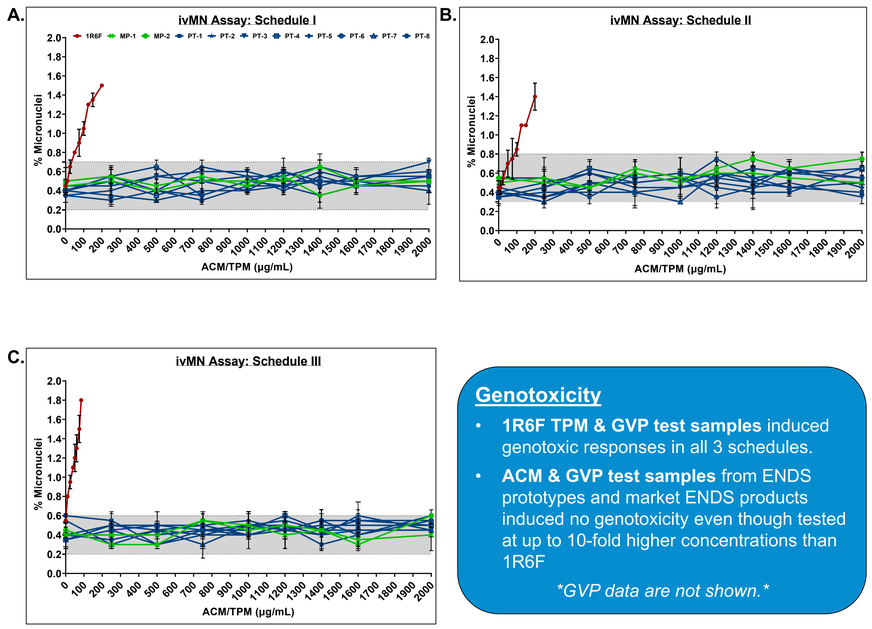


Cytotoxicity

- **1R6F TPM & GVP test samples** induced dose-dependent reductions in viability with IC_{50} values of 96.0 µg/mL and 207.8 µg/mL, respectively.
- **ACM & GVP test samples** from ENDS market products & ENDS prototypes induced no reduction in viability.

GVP data are not shown.

Figure 3. *In vitro* Micronucleus (ivMN) Assay. TPM/ACM and GVP test samples were tested at concentrations up to 200 µg/mL (1R6F) and up to 2000 µg/mL (ENDS). Test samples were assessed across three schedules and representative results for TPM/ACM test samples are shown below for **A.** Schedule I (short term without (-S9) metabolic activation); **B.** Schedule II (short term +S9); **C.** Schedule III (long term -S9). Data are normalized to mass.

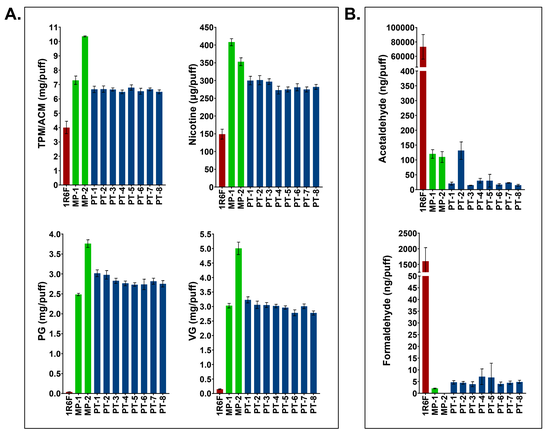


Genotoxicity

- **1R6F TPM & GVP test samples** induced genotoxic responses in all 3 schedules.
- **ACM & GVP test samples** from ENDS prototypes and market ENDS products induced no genotoxicity even though tested at up to 10-fold higher concentrations than 1R6F

GVP data are not shown.

Figure 4. Chemistry Analysis. A. TPM/ACM test samples were analyzed for nicotine, propylene glycol (PG), glycerol (VG), and water (not shown). **B.** GVP test samples were analyzed for select carbonyls including acetaldehyde, formaldehyde, acrolein, and crotonaldehyde. Data represent the average of three collections.



Chemistry Analysis

- The average amounts of nicotine, PG, and VG for all ENDS products were higher than that of 1R6F on a per puff basis.
- Measurement of select carbonyls revealed significantly lower levels of acetaldehyde and formaldehyde for all ENDS products. Levels of acrolein and crotonaldehyde were below the detection limit for all ENDS products (data not shown).

Conclusions

- **1R6F was considered mutagenic and genotoxic, while all tested ENDS products were considered non-mutagenic and non-genotoxic in the AMES assay and ivMN assay, respectively; these results suggest ENDS products have a lower genotoxic hazard compared to traditional combustible products.**
- **1R6F was considered cytotoxic, while none of the tested ENDS products induced cytotoxic effects in the NRU assay, suggesting a lower cytotoxic hazard for ENDS products.**
- **Chemical analyses demonstrated that ACM and GVP test samples from the ENDS test items generated substantially lower levels of harmful and potentially harmful chemicals (HPHCs) compared to 1R6F which parallels the substantially lower biological activity of ENDS in the *in vitro* assays.**
- **Collectively, these data indicate that the ENDS products tested in this study present a potentially reduced harm alternative compared to combustible tobacco products.**

References

1. ISO 20778:2018 Cigarettes — Routine analytical cigarette smoking machine — Definitions and standard conditions with an intense smoking regime.
2. ISO 20768:2018 - Vapour Products — Routine Analytical Vaping Machine — Definitions and Standard Conditions.
3. OECD Guideline for Testing Chemicals, Bacterial Reverse Mutation Assay No. 471, adopted on 26 June 2020.
4. OECD Guideline for Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Test, No. 129, adopted on 20 July 2010.
5. OECD Guideline for In Vitro Mammalian Cell Micronucleus Test, No. 487, adopted on 3 July 2023.

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