Genotoxicity Assessment of Combustible Cigarette, Heated Tobacco Product (HTP) and Electronic Nicotine Delivery Systems (ENDS) Aerosols and Modern Oral (MO) Nicotine Product Extracts in the Ames and *In Vitro* Micronucleus Assays

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Abstract

In vitro **genotoxicity assessment of tobacco products is an essential piece of the premarket tobacco product application (PMTA) process. Next generation tobacco products (NGPs) include Electronic Nicotine Delivery Systems (ENDS), Heated Tobacco Products (HTP) and Modern Oral (MO) nicotine products. The genotoxicity of combustible cigarette (CC), HTP and ENDS aerosols and MO extracts was determined using the Ames and *in vitro* micronucleus (IVMN) assays. Test samples from CC, HTP and ENDS included pad-collected total particulate matter (TPM: CC & HTP) or aerosol collected material (ACM: ENDS) and gas vapor phase (GVP) preparations. MO products were tested using complete artificial saliva (CAS) extracts. TPM/ACM and GVP were tested either separately or combined 1:1 (v:v). The Ames assay utilized *Salmonella* tester strains TA98, TA100, TA102, TA1535 & TA1537 (±S9). For IVMN, the three standard exposure schedules (i-iii) were performed, with an additional exposure (iv: 24-hr, -S9, with 24-hr recovery) for HTP and MO samples. CC TPM and combined TPM+GVP were genotoxic in both the Ames and IVMN. ENDS and MO tested negative in both the Ames and IVMN, while the HTP was non-mutagenic in the Ames but genotoxic in the IVMN, albeit less toxic (slope comparison, p<0.0001) at ~10X higher delivered nicotine concentrations compared to CC. Overall, the results from this series of studies provide data supporting the tobacco harm reduction paradigm, with a decrease in genotoxicity over a correlated panel of different representative tobacco product types along a decreasing risk continuum compared to combustible cigarettes.**

Materials and Methods

Test Item Conditioning:

- **CC and HTP consumables were conditioned for at least 48 hr at 22 ± 1°C, 60 ± 3% relative humidity (ISO 3402, 1999)**
- **ENDS were stored at RT, in their normal packaging, prior to use. Power units were fully charged prior to use**
- **MO were stored frozen (< 18°C), thawed at 1-8°C for a minimum of 24 hr and then allowed to equilibrate to RT for a minimum of 2 hr prior to CAS extraction**

TPM / ACM and GVP Generation (Figure 1):

- **CC TPM: 55 mL puff, 2 sec puff, 30 sec interval; 100% vent blocking (ISO 20778, 2018) collected on 1 x 92 mm pad and extracted in DMSO at 10 mg/mL**
- **HTP TPM: 55 mL puff, 2 sec puff, 30 sec interval collected on 1 x 92 mm pad and extracted in DMSO at 100 mg/mL**
- **ENDS ACM: 80 mL puff, 4 sec puff, 15 sec interval collected on 1 x 92 mm pad, extracted in DMSO at 50 mg/mL**
- **CC, HTP & ENDS GVP collected concurrently using impinger containing 15 mL calcium-magnesium-free (CMF) PBS. Final volume adjusted to match [TPM / ACM] at mg TPM / ACM Equivalents / mL**
- **TPM & GVP fractions tested seperately (ENDS) or combined (1:1, v/v; CC & HTP); applied to cultures within 1 hr of generation**

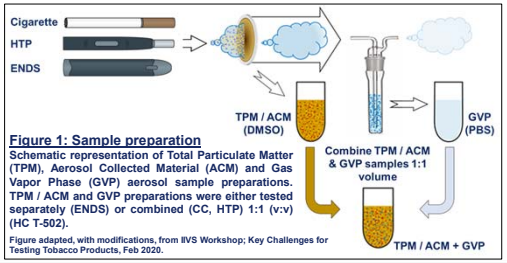

Figure 1: Sample preparation
Schematic representation of Total Particulate Matter (TPM), Aerosol Collected Material (ACM) and Gas Vapor Phase (GVP) aerosol sample preparations. TPM / ACM and GVP preparations were either tested separately (ENDS) or combined (CC, HTP) 1:1 (v:v) (HC T-502).

Figure adapted, with modifications, from IIVS Workshop; Key Challenges for Testing Tobacco Products, Feb 2020.

Results

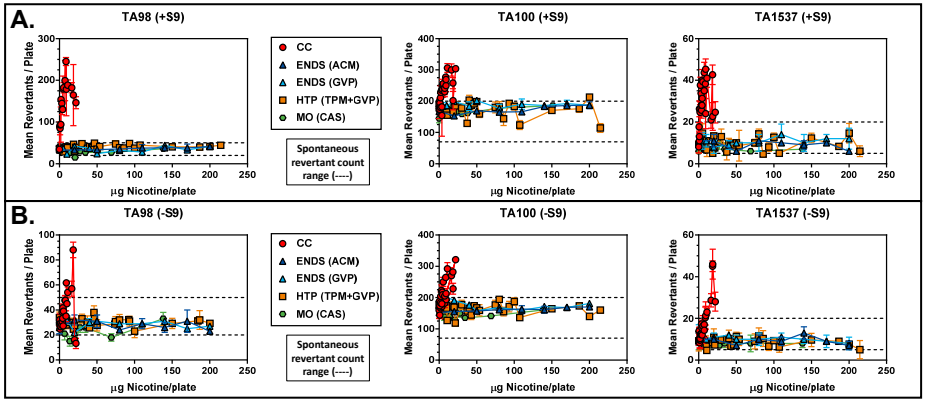

Figure 2: ENDS, HTP and MO not mutagenic in the Ames Assay. Ames Assay Results (preincubation method) in the presence (A) and absence (B) of metabolic activation (±S9), methods based on HC T-501 and OECD 471 guidelines. Results from *Salmonella* tester strains TA98, TA100 and TA1537 are displayed. Data not shown for *Salmonella* tester strains TA102 and TA1535 (±S9) since no mutagenic activity was observed from any of the test items. Combustible cigarette (TPM+GVP) was mutagenic in the three strains shown, with evidence of toxicity at the higher doses as indicated by the decreases in revertant counts at the higher doses. The ENDS (ACM and GVP), HTP (TPM+GVP) and MO (CAS) test items showed no mutagenic activity in all five tester strains, at doses considerably higher than the CC. ENDS, HTP and MO revertant counts consistently fell within the historic range of spontaneous revertant counts (- - - -) for each respective tester strain. Tested doses are based on the amount of nicotine (µg / plate) from the different sample preparations (TPM, ACM, GVP and CAS extracts) in order to allow the concurrent presentation of the results obtained from multiple studies.

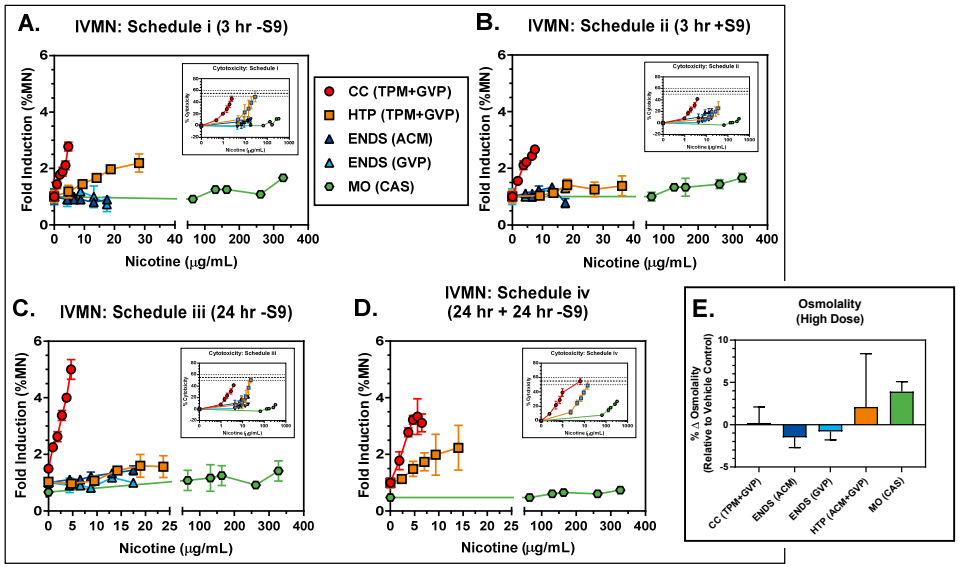

Figure 3: ENDS and MO non-genotoxic in the IVMN Assay. *In Vitro* Micronucleus (IVMN) assay results (A - D) observed under four exposure schedules (i – iv). IVMN methods (without cytochalasin B) utilized in this series of studies were based on HC T-503 and OECD 487 guidelines. Schedule iv, 24 hr exposure (-S9) with a 24 hr recovery prior to harvesting, referenced from Thorne *et al* (2019). Insert graphs (A – D) display the observed cytotoxicity (based on relative increase in cell count: RICC) over the same dose range. Combustible cigarette (TPM+GVP) displayed genotoxicity in all four exposure schedules, indicated by the dose related increase in micronuclei (MN) induction. HTP also displayed dose related increases in MN induction in two of the four testing schedules (i and iv). ENDS (ACM and GVP) and MO (CAS) exposures resulted in no overall genotoxicity. Changes in Osmolality (E) were determined to rule out any potential effects on MN induction by osmotic stress. Minimal changes in osmolality were observed for all test items, with MO showing the largest mean change of 4%.

Materials and Methods (cont.)

CAS Extraction

- **CAS contained mucin, α-amylase, lysozyme and acid phosphatase (Chou & Hee, 1994)**
- **MO product ground to ~ 4mm particle size and extracted in CAS for 2 hr at 300 mg/mL, shaking at 250 rpm at 37 ± 1°C**
- **MO extract centrifuged 2500 rpm, 15 min at RT, supernatant filtered (1.6 µm) then filter sterilized (0.22 µm)**

Ames Assay: Preincubation (OECD 471, HC T-501)

- ***Salmonella* strains TA98, TA100, TA102, TA1535 & TA1537 (±S9)**
- **Aroclor 1254-induced Sprague Dawley Rat liver S9 (Moltox, Boone, NC, USA)**
- **Individual strains mixed with test item, 5% S9-mix (+S9) or PBS (-S9) and incubated 20 ± 2 min at 37 ± 1°C**
- **Molten top agar added to exposure mix and poured on minimal agar plates and incubated 48 – 72 hr at 37 ± 1°C**
- **Revertant colonies counted with an automated colony counter**

***In Vitro* Micronucleus Assay (OECD 487, HC T-503)**

- **CHO-WBL , seeded into tissue culture flasks at ~1x10^5 cells / mL in Ham's F-12 media, incubated at 37 ± 1°C [5% (v/v) CO_2] for ~24 hr prior to exposure**
- **Aroclor 1254-induced or Phenobarbital Benzoflavone-induced Sprague Dawley Rat liver S9 (Moltox, Boone, NC, USA)**
- **Duplicate flasks exposed to TPM / ACM and GVP either separately (ENDS) or combined (CC, HTP) or MO CAS extracts under four exposure schedules:**
  - **Schedule i: 3 hr exposure (-S9) + 21 hr recovery (All test items)**
  - **Schedule ii: 3 hr exposure (+S9) + 21 hr recovery (All test items)**
  - **Schedule iii: 24 hr exposure (-S9) with no recovery (All test items)**
  - **Schedule iv: 24 hr exposure (-S9) + 24 hr recovery (CC, HTP, MO)**
- **After exposure and recovery, slides are prepared for manual scoring. Minimum of 2000 cells scored per concentration.**

Studies were conducted under contract at Labstat International Inc., Kitchener, ON Canada

Summary & Conclusions

- **The CC was mutagenic in tester strains TA98, TA100 and TA1537 (±S9) (Figure 2).**
- **ENDS, HTP and MO were non-mutagenic in all tester strains, with no observed dose related revertant increase and plate counts falling within the spontaneous revertant historical ranges (Figure 2).**
- **The CC was genotoxic in all four IVMN exposure schedules with dose dependent increases in MN induction (Figure 3).**
- **The HTP was genotoxic in IVMN exposure schedules i and iv; at tested doses 2 – 10X's higher than CC (Figure 3).**
- **ENDS and MO were non-genotoxic in all four IVMN exposure schedules (Figure 3).**
- **The Tobacco Harm Reduction paradigm for Next Generation Tobacco Products places combustible cigarettes as the most harmful. Results from this study add to the weight of evidence that would place HTP, ENDS and MO products downstream of combustible cigarettes along this spectrum of potential harm.**

References

- **Chou, C. C., and Hee, S. S. Bioassay-driven analysis of chewing tobacco extracts (1994). *Environ. Toxicol. Chem.* 13, 1177-86.**
- **Health Canada Official Method T-501; Bacterial Reverse Mutation Assay for Mainstream Tobacco Smoke (2017)**
- **Health Canada Official Method T-502; Neutral Red Uptake Assay for Mainstream Tobacco Smoke (2017)**
- **Health Canada Official Method T-503; *In Vitro* Micronucleus Assay for Mainstream Tobacco Smoke (2017)**
- **OECD 471; Bacterial Reverse Mutation Test (2020)**
- **OECD 487; *In Vitro* Mammalian Cell Micronucleus Test (2016)**
- **Thorne *et al*. Genotoxicity evaluation of tobacco and nicotine delivery products: Part Two. *In vitro* micronucleus assay (2019) *Food Chem. Tox.* 132, 110546.**

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