Tobacco Harm Reduction: *In Vitro* Toxicological Assessment of Next Generation Tobacco and Nicotine Products versus Combustible Cigarettes and Smokeless Tobacco

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Abstract

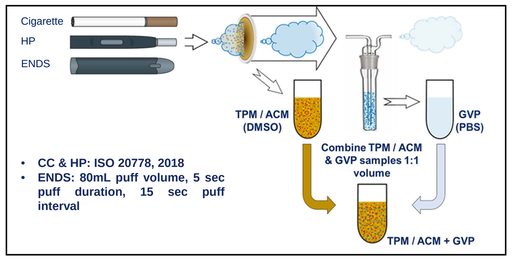
Next generation tobacco and nicotine (NGP) products, which include Electronic Nicotine Delivery Systems (ENDS), Heated Tobacco Products (HP), and Nicotine Pouch (NP) products, provide alternative, potentially less harmful, nicotine products to adult smokers and nicotine consumers wanting to transition from combustible and other tobacco containing products.

NGPs must undergo the premarket tobacco product application (PMTA) process and obtain a marketing granted order (MGO) from FDA Center of Tobacco Products (CTP) to become authorized for marketing. Currently, FDA-CTP PMTA submissions require comprehensive listings of product ingredients and components, harmful and potentially harmful constituents (HPHC), product stability and information on potential health risks including risk assessments, *in vitro* toxicological, and clinical testing to establish a Weight of Evidence (WoE) to determine if the marketing of an NGP is appropriate for the protection of the public health (APPH).

This assessment will examine the *in vitro* toxicological testing of representative ENDS, HP and NP products (all menthol or mint flavor varieties) through three standard genotoxicity and cytotoxicity assays and compared to combustible cigarettes (CC) and traditional smokeless tobacco (ST). The three standard regulatory toxicology assays utilized include the Ames (mutagenicity), *in vitro* micronucleus (ivMN: genotoxicity), and neutral red uptake (NRU: cytotoxicity) following respective Health Canada (HC) and OECD guidelines. Test samples from CC, HP, and ENDS included pad-collected total particulate matter (TPM: CC & HP) or aerosol collected material (ACM: ENDS), all combined with their respective gas vapor phase (GVP) preparations as well as combustible cigarette whole smoke (WS) and whole aerosol (WA) from ENDS and HP (NRU). NP products with either tobacco-derived (TDN) or synthetic (SYN) nicotine and ST (snus) were tested using complete artificial saliva (CAS) extracts in all three assays.

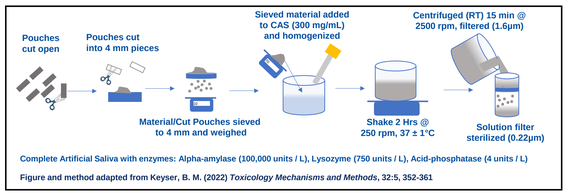
Materials and Methods

Figure 1: CC, HP & ENDS Sample Generation (Ames & ivMN)



- CC & HP: ISO 20778, 2018
- ENDS: 80mL puff volume, 5 sec puff duration, 15 sec puff interval

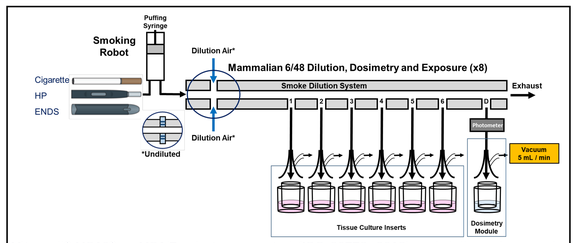
Figure 2: Complete Artificial Saliva (CAS) Extracts (AMES, ivMN & NRU)



Complete Artificial Saliva with enzymes: Alpha-amylase (100,000 units / L), Lysozyme (750 units / L), Acid-phosphatase (4 units / L)

Figure and method adapted from Keyser, B. M. (2022) *Toxicology Mechanisms and Methods*, 32:5, 352-361

Figure 3: CC, ENDS & HP Whole Aerosol Exposure (NRU)



Vitrocell® VC10® or VC1/7 robots generated (ISO 20778, 2018) and delivered aerosols to the Mammalian 6/48 aerosol dilution and exposure system, with up to 7 concurrent doses plus a clean air control. The dosimetry modules allowed the capture and quantification of deposited aerosol constituents (nicotine, glycerol and carbonyls).

Figure adapted, with modifications, from Keyser *et. al.* (2019) *Toxicology Reports*, 6, 1281-1288.

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Results

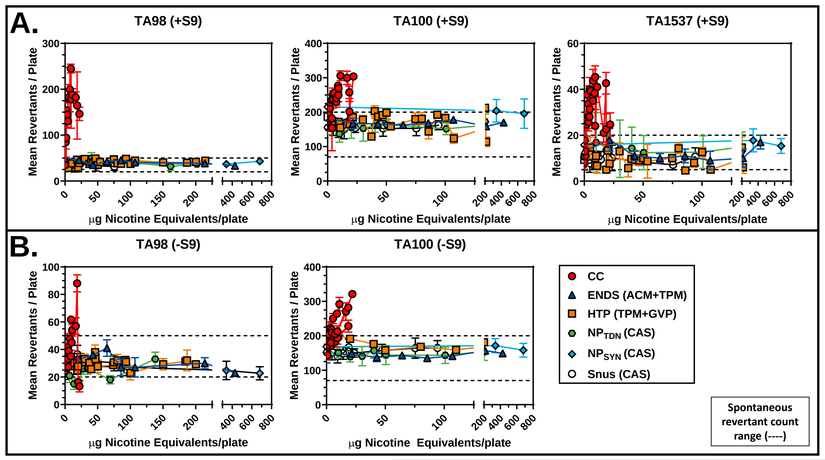


Figure 4: CC Mutagenic, NGPs Non-Mutagenic in the Ames Assay.
Ames assay results (preincubation method) in the (A) presence (+S9) and (B) absence (-S9) of metabolic activation. Methods based on HC T-501 and OECD 471 guidelines. Results from *Salmonella* tester strains TA98 (±S9), TA100 (±S9) and TA1537 (+S9) are displayed since activity typically not observed in tester strains TA102 (±S9), TA1535 (±S9) and TA1537 (-S9). CC was mutagenic in the three strains shown, with dose related increases exceeding the historical spontaneous revertant count range. ENDS, HP, NP and Snus test items consistently showed no mutagenic activity in all five tester strains (revertant counts consistently fell within the spontaneous revertant historic range for each respective tester strain), at nicotine equivalent doses (µg/plate) considerably higher (>10X) than the CC. Results (Mean ± SD) from three (3) independent experiments.

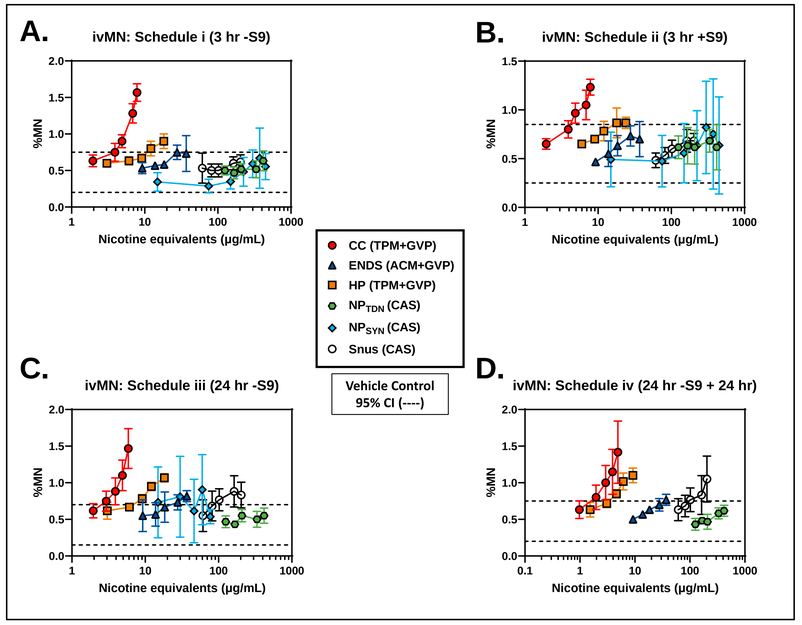
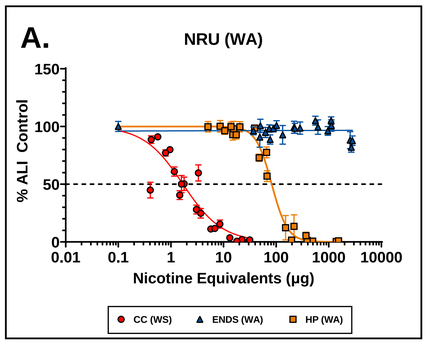


Figure 5: CC and HP Genotoxic, ENDS and NP Non-Genotoxic in the ivMN Assay.
In Vitro Micronucleus (ivMN) assay results (A - D) observed under four exposure schedules (i – iv). ivMN methods (without cytochalasin B) based on HC T-503 and OECD 487 guidelines. Schedule iv, 24 hr exposure (-S9) with a 24 hr recovery prior to harvesting, referenced from Thorne *et al* (2019). CC (TPM+GVP) displayed genotoxicity in all four exposure schedules, indicated by the dose related increase in micronuclei (MN) induction. HP also displayed genotoxicity in Schedule iv only (meeting all acceptance criteria for a positive response). Snus did see dose related increases in MN induction in schedules iii and iv; however, all acceptance criteria for a positive response were not met. ENDS (ACM+GVP) and NP (CAS) exposures resulted in no overall genotoxicity. NP_{SYN} was not tested in Schedule iv.



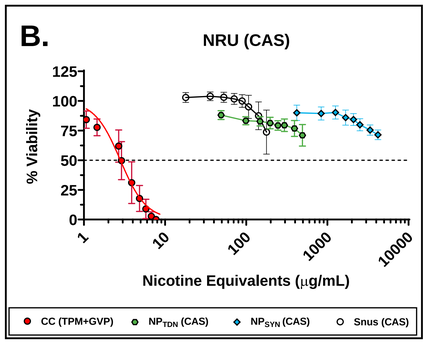


Figure 6: CC and HP Cytotoxic; ENDS and NP Non-Cytotoxic in NRU Assay.
Whole aerosol (WA) NRU results (A) for CC, ENDS and HP and (B) for CC (TPM+GVP), NP (CAS) and Snus (CAS). CC (WA and TPM+GVP) resulted in cytotoxicity with calculated IC_{50} values based on delivered nicotine equivalents of 1.7 µg Nicotine equivalents (WA) and 3 µg Nicotine equivalents per mL (TPM+GVP), respectively. HP (WA) was also observed to be cytotoxic but with an IC_{50} of 82.5 µg Nicotine equivalents, which is considerably higher (> 45X) than CC (WA). No indication of cytotoxicity was observed for ENDS, NP and Snus comparator.

Materials and Methods (cont.)

Ames Assay:

- Preincubation method (HC-501; OECD 471)
- *Salmonella* strains TA98, TA100, TA102, TA1535 & TA1537 (±S9)
- Phenobarbital-5,6 Benzoflavone-induced Sprague Dawley Rat liver S9

***In Vitro* Micronucleus (ivMN) Assay:**

- HC T-503; OECD 487
- CHO or V79 mammalian cells
- Phenobarbital-5,6 Benzoflavone-induced Sprague Dawley Rat liver S9
- Three or four exposure schedules:
  - Schedule i: 3 hour exposure (-S9) + 21 hour recovery
  - Schedule ii: 3 hour exposure (+S9) + 21 hour recovery
  - Schedule iii: 24 hour exposure (-S9) with no recovery
  - Schedule iv: 24 hour exposure (-S9) + 24 hour recovery (Thorne *et. al.*, 2019)
- Micronuclei scoring by manual or flow cytometry (Litron Labs MicroFlow®) methods

Neutral Red Uptake (NRU) Assay:

- Plate Method (CC, NP, ST)
  - HC-502; OECD 129
  - CHO or Balb/c 3T3 mammalian cells
- Whole Aerosol Method (CC, HP, ENDS)
  - Keyser *et. al.* 2024a; Keyser *et. al.* 2024b
  - H292 mammalian cells

All studies presented were conducted under contract at Labstat International Inc., Kitchener, ON Canada and Labcorp, Harrogate, UK

Summary and Conclusions

- The representative results presented here are from several studies across the different test items (CC, ENDS, HP, NP and Snus) and biological endpoints (mutagenicity, genotoxicity and cytotoxicity).
- CC was consistently found to be mutagenic in strains TA98 (±S9), TA100 (±S9) and TA1537 (+S9) (Ames: Figure 4).
- NGPs and the Snus comparator were non-mutagenic in all tester strains (Ames: Figure 4).
- CC was found to be genotoxic, inducing micronuclei across the four ivMN exposure schedules and HP was deemed genotoxic in Schedule iv only (ivMN: Figure 5).
- ENDS and oral test items were non-genotoxic, not meeting all the acceptance criteria for a positive response (ivMN: Figure 5).
- CC (WS and TPM+GVP) were found to be cytotoxic (IC_{50}: 1.7 µg nicotine equivalents and 3 µg nicotine equivalents / mL, respectively) (NRU: Figure 6).
- HP (WA) was found to be cytotoxic (IC_{50}: 82.5 µg nicotine equivalents) at doses considerably higher (> 45X) than CC (WS) (NRU: Figure 6).
- ENDS (WA), NP (CAS) and Snus (CAS) test items were non-cytotoxic (NRU: Figure 6).
- Overall, the *in vitro* test battery results presented here add to the WoE that non-combustible NGPs have reduced *in vitro* toxicity (mutagenicity, genotoxicity, and cytotoxicity) compared to CC. These results support the tobacco harm reduction (THR) paradigm, where combustible cigarettes represent the most harmful tobacco product and next generation tobacco products (e.g., HP, ENDS and NP) fall along a decreasing risk continuum.

References

- CORESTA Guide 11 (2020) Technical Guide for Sample Handling of Smokeless Tobacco and Smokeless Tobacco Products
- Health Canada Method T-501 (2017) Bacterial Reverse Mutation Assay for Mainstream Tobacco Smoke
- Health Canada Method T-502 (2017) Neutral Red Uptake Assay for Mainstream Tobacco Smoke
- Health Canada Method T-503 (2017) *In Vitro* Micronucleus Assay for Mainstream Tobacco Smoke
- ISO 3402 (1999) Tobacco and Tobacco Products – Atmosphere for Conditioning and Testing
- OECD Test Guideline 129 (2010) Guidance Document on Using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests
- ISO 20778 (2018) Routine Analytical Cigarette Smoking Machine, Definitions and Standard Conditions with an Intense Smoking Regime
- Keyser *et. al.* (2024a) *Toxicology* 504 153801
- Keyser *et. al.* (2024b) *Toxics* 12, 129
- OECD Test Guideline 471 (2020) Bacterial Reverse Mutation Test
- OECD Test Guideline 487 (2016) *In Vitro* Mammalian Cell Micronucleus Test
- Thorne *et al.* (2019) *Food Chemical Toxicology* 132, 110546

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