Title: Genotoxicity Assessment of Heated Tobacco Product and Combustible Cigarette Aerosols in the Ames and In Vitro Micronucleus Assays

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Abstract

In vitro **toxicological methods are used to assess the biological activities of combustible and next generation tobacco products (NGP), including Heated Tobacco Products (HTP). To determine the genotoxic potential of aerosols generated from four HTP (glo™) styles, a marketed HTP comparator and three combustible cigarettes (CC), the bacterial reverse mutation (Ames) and *in vitro* micronucleus (IVMN) assays were conducted using test sample preparations of total particulate matter (TPM) combined with gas vapor phase (GVP). Ames preincubation assays utilized tester strains TA98, TA100, TA1535, TA1537 and TA102 (±S9). For the IVMN, CHO cells were exposed under four different schedules.**

In the Ames assay, all CC were mutagenic based on positive responses in 3 of 5 test strains, while the HTP were negative across all strains and test conditions (±S9) when tested at nicotine equivalent doses up to 10-fold greater than CC. In the IVMN assay, all CC produced positive genotoxic responses in all exposure schedules as indicated by dose-related increases in micronuclei. In contrast, genotoxic responses of some HTP were observed only in certain schedules when testing nicotine-equivalent doses that ranged from 4.5-10x of that of the CC. These results add to the weight of evidence from multiple studies on the harm reduction potential of HTPs when compared to CC, supporting the tobacco harm reduction paradigm of NGPs.

Materials and Methods

Generation/Preparation of Test Matrices:

Four different flavor variants of a single HTP product, a HTP market comparator and 3 CC were assessed under GLP conditions at a contract research laboratory using standard nonclinical regulatory toxicology procedures. For each test article, aerosol fractions (TPM+GVP) were generated and combined in a 1:1 ratio forming a single test matrix as described below and shown in Figure 1.

The TPM for all products were generated using an automated rotary smoking machine and collected on 92 mm Cambridge filter pads. The smoking regimen for CC was HCI (T-502) and HTP test items were smoked according to a Modified HCI. For all test articles, mainstream GVP was bubbled into a cooled glass impinger containing phosphate-buffered saline (PBS). DMSO was used to elute the TPM from the pads to a stock concentration of 10 mg/mL. The volume of the trapped GVP was adjusted with PBS to achieve 10 mg TPM equivalent/mL.

Figure 1: TPM+GVP Test Sample Generation

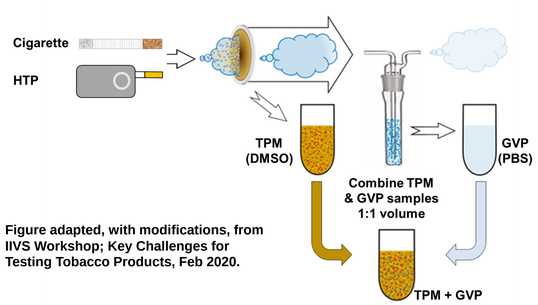


Figure adapted, with modifications, from IIVS Workshop; Key Challenges for Testing Tobacco Products, Feb 2020.

Results

Table 1: Overall Ames and *iv*MN Results

Test Article	Overall Ames Result (if positive, strain ±S9)	Overall *iv* MN Results
glo neo neoCLICK	Negaive	Genotoxic (Schedule I)
glo neo Fresh Menthol	Negative	Genotoxic (Schedule II and IV)
glo neo Smooth Menthol	Negative	Genotoxic (Schedule IV)
glo neo Smooth Tobacco	Negative	Non-Genotoxic
Market HTP	Negative	Genotoxic (Schedules I and IV)
Market Nonmenthol CC	Mutagenic (TA98±S9, TA100±S9, TA1537±S9	Genotoxic (Schedule I, II, III, IV)
Market Menthol CC	Mutagenic (TA98±S9, TA100±S9, TA1537±S9	Genotoxic (Schedule I, II, III, IV)
Kentucky Reference 1R6F	Mutagenic (TA98±S9, TA100±S9, TA1537+S9	Genotoxic (Schedule I, II, III, IV)

Figure 2: Ames Data for Select Strains

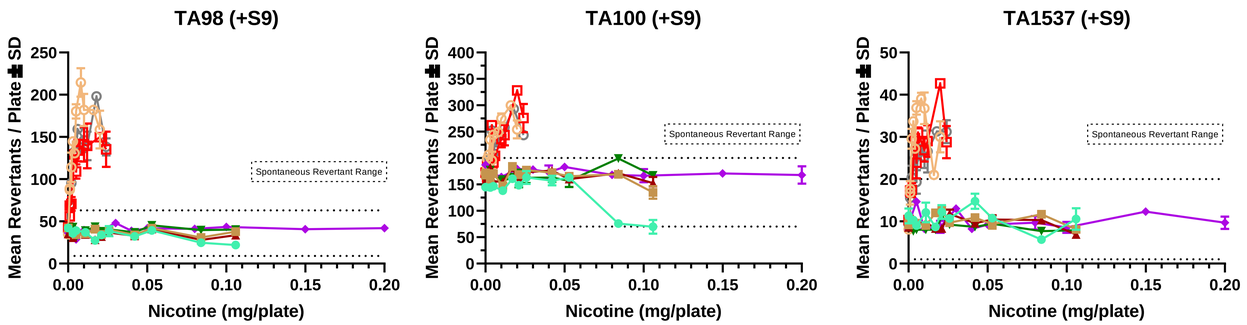
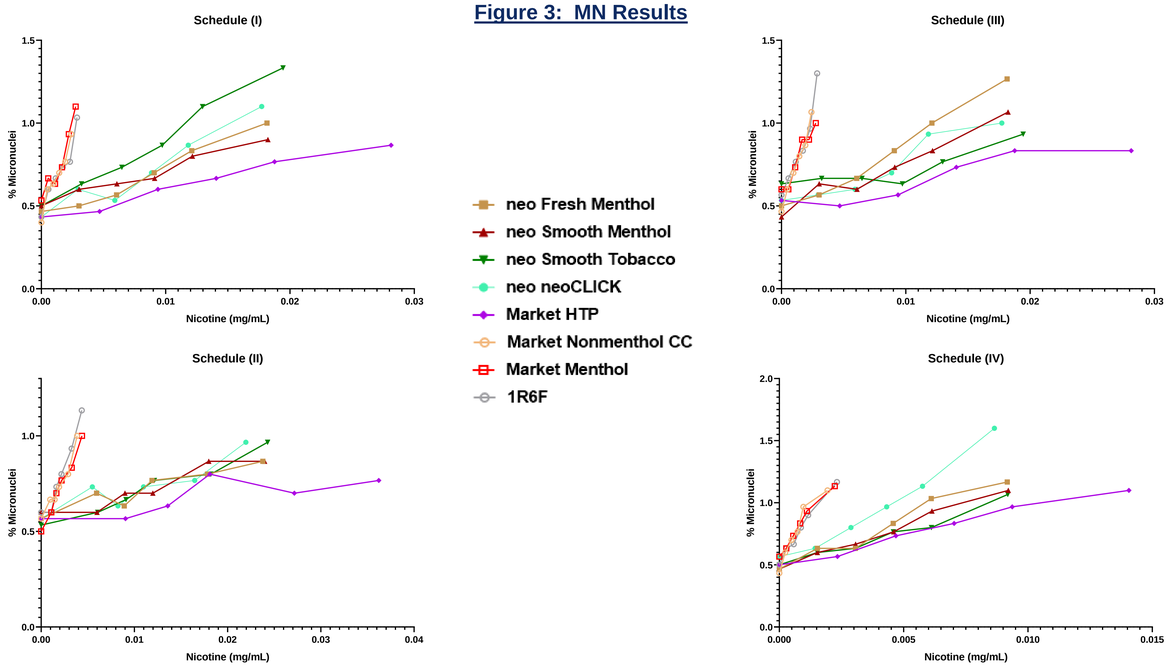


Figure 3: MN Results



Materials and Methods (cont)

Combined samples (1:1, v/v) of the TPM+GVP were used for Ames and *iv*MN exposures. The GVP samples were tested in each of the biological assays (Ames and *iv*MN) within 1 hour of generation.

In order to avoid potential artifactual positive response, the highest concentration of test article assessed in these assays was limited to one that did not produce excessive toxicity (as indicated by a decrease in background lawn for Ames and %RICC <60% at the highest dose tested for *iv*MN), precipitation in the culture medium, or marked changes in pH or osmolality.

Ames bacterial reverse mutation assay:

TPM+GVP test sample exposures were conducted to the principles of OECD 471 and HC T-501, using 5 bacterial (*Salmonella typhimurium)* strains: TA98, TA100, TA1535, TA1537, and TA102; each with and without metabolic activation (5% S9 mix). Overall assay results for each test article are summarized in Table 1. Results for select strains and conditions are reported in terms of the average number of observed revertants per plate following exposure to the test sample (see Figure 2).

***In vitro* micronucleus genotoxicity assay:**

In alignment with the principles of OECD 487 and HC T-503, TPM+GVP test sample genotoxicity was determined as follows. CHO cells were exposed to the test sample in one of three conditions: ± S9 with short 3h exposure and 21h recovery or a longer 24h exposure
(-S9). Results for the *iv*MN assay are presented as (Mean) %.

Conclusions

Ames:

- **For the HTP test articles, mutagenicity was not observed in any of the bacterial strains tested, with or without exogenous metabolic activation, at test concentrations up to the maximum deliverable concentration (10000 µg/plate of TPM+GVP) as defined by OECD**

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- **Mutagenic responses were observed for the TPM + GVP test samples generated from the combustible cigarette in strains TA98 (+S9), TA100 (±S9), & TA1537 (±S9) (see Table 1).**

***iv*MN:**

- **All combustible cigarettes produced positive genotoxic responses under all exposure schedules as indicated by dose-related increases in micronuclei. In contrast, some HTPs demonstrated positive genotoxicity under certain treatments. Additionally, the nicotine-equivalent concentration for positive HTP genotoxicity was 4.5-10x higher than for the combustible cigarette.**

- **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 HTPs downstream of combustible cigarettes along a decreasing risk continuum.**