

In Vitro Toxicological Assessment of Modern Oral Nicotine Product Extracts in the Ames, Micronucleus, and Neutral Red Uptake Assays

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Background

Current next generation tobacco products (NGPs) include Modern Oral (MO) nicotine products containing nicotine, either synthetic or derived from tobacco, and tobacco leaf. *In vitro* toxicological assessments of tobacco products are a significant part of the US FDA's premarket tobacco product application (PMTA) process, providing data for a weight of evidence (WoE) approach to determine if the marketing of a new MO tobacco product is appropriate for the protection of the public health (APPH). Data for these products is important due to the limited information for this product class and their accelerating growth in the market.

Methodology

The *in vitro* toxicological assessment of a marketed MO nicotine pouch product, spanning a variety of flavors and nicotine levels, along with a marketed MO product and a marketed combustible cigarette (CC) was conducted using a battery of well-established assays. These assays included the Bacterial Reverse Mutation (Ames), *In Vitro* Micronucleus (ivMN), and Neutral Red Uptake (NRU) assays to assess mutagenicity, genotoxicity, and cytotoxicity, respectively. The MO test products and the MO market comparator were tested using complete artificial saliva (CAS) extract sample preparations.

MO product sample extracts were prepared at a concentration of 300 mg product (mixture of pouch content and material) per milliliter CAS. On each experimental day, cigarettes were smoked under ISO 20778 (2018) puffing parameters. Pad-collected total particulate matter (TPM) from mainstream smoke was extracted in DMSO at 24 mg/mL and gas vapor phase (GVP) was captured in calcium and magnesium free buffered saline (CMF-PBS). Cigarette smoke TPM and GVP sample fractions were combined on a 1:1 (v:v) basis to achieve a final concentration of 12 mg/mL TPM + GVP. All test sample preparations were analyzed for nicotine (MO/CAS) and carbonyls (CC TPM+GVP).

The Ames assay utilized the preincubation method in *Salmonella* tester strains TA98, TA100, TA102, TA1535, and TA1537 with and without metabolic activation ($\pm$ S9). In the ivMN and NRU assays, V79 and BALB/c 3T3 cells, respectively, were exposed to a range of concentrations of either CAS or TPM+GVP preparations. In the ivMN, experiments were conducted under the standard three (3) exposure schedules (short-term with S9, short- and long-term without S9) prior to micronuclei scoring via flow cytometry using Litron Laboratories MicroFlow[®] kits. For each assay, three independent sample preparations and experimental trials were conducted.

The concentration ranges used were dependent on solvent, osmotic effects, and cytotoxicity limitations for each assay type, as outlined in their respective method guidelines. All assays were conducted under GLP following the corresponding OECD and Health Canada guidelines.

Results

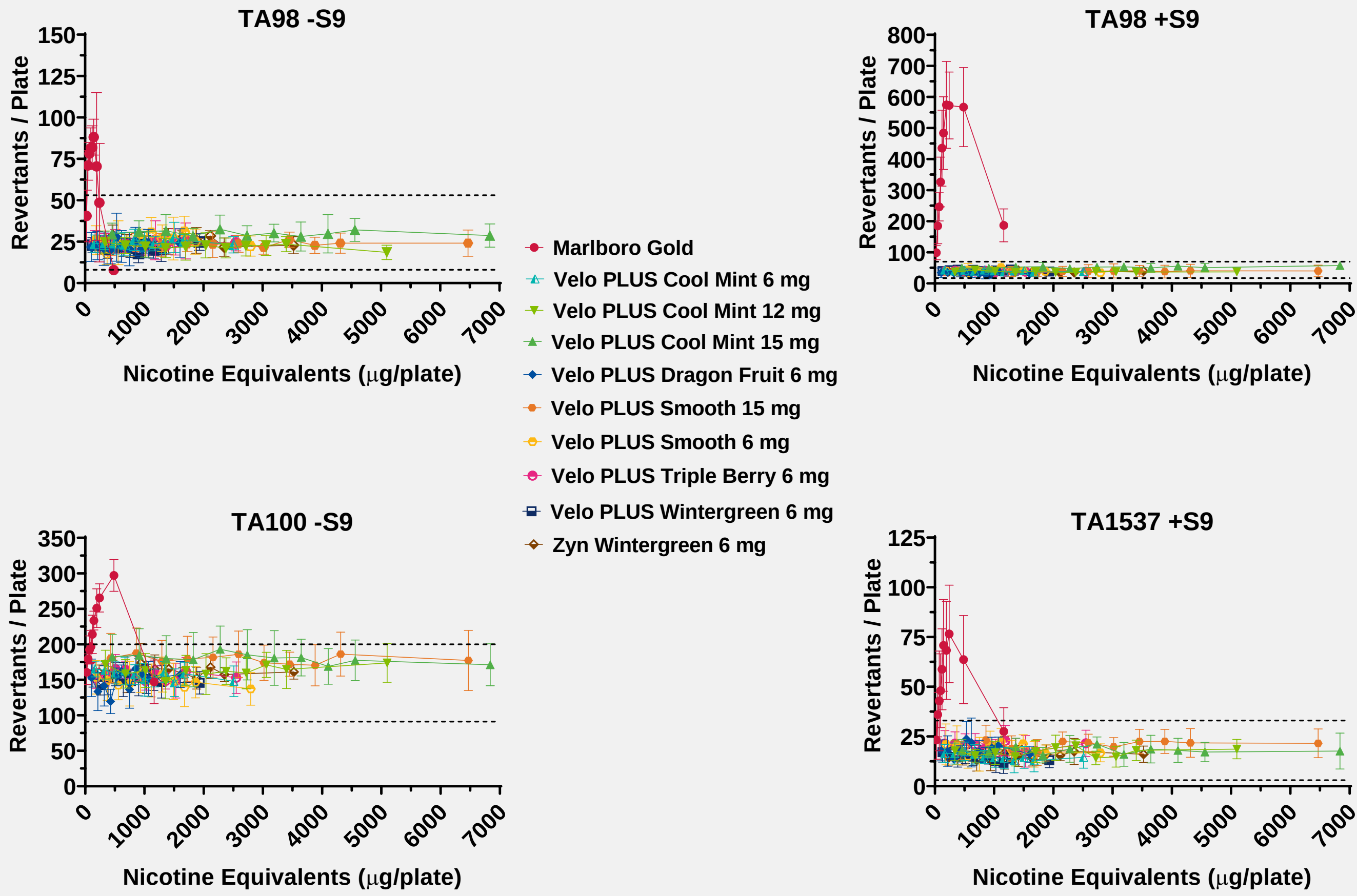


Figure 1. Ames Data for Select Strains ($\pm$ S9). Mutagenic responses were observed for the Combustible in TA98 $\pm$ S9, TA100 -S9, TA1537 +S9; MO test articles were negative in all stains. TPM+GVP test sample (combustible) or CAS extracts exposures using 5 bacterial (*Salmonella typhimurium*) strains: TA98, TA100, TA1535, TA1537, and TA102; each with and without metabolic activation (5% S9 mix). Results for mutagenic responses are reported in terms of the average number of observed revertants per plate (indication of mutagenicity) following exposure to the test sample. Dotted lines are historical spontaneous revertant counts for each strain and condition. Triplicate plates per concentration, n=3, results are mean $\pm$ SD. Positive criteria: Concentration-related increase in revertants; Dunnet's test significant (p<0.01) increase in revertants; and reproducible increase in revertants.

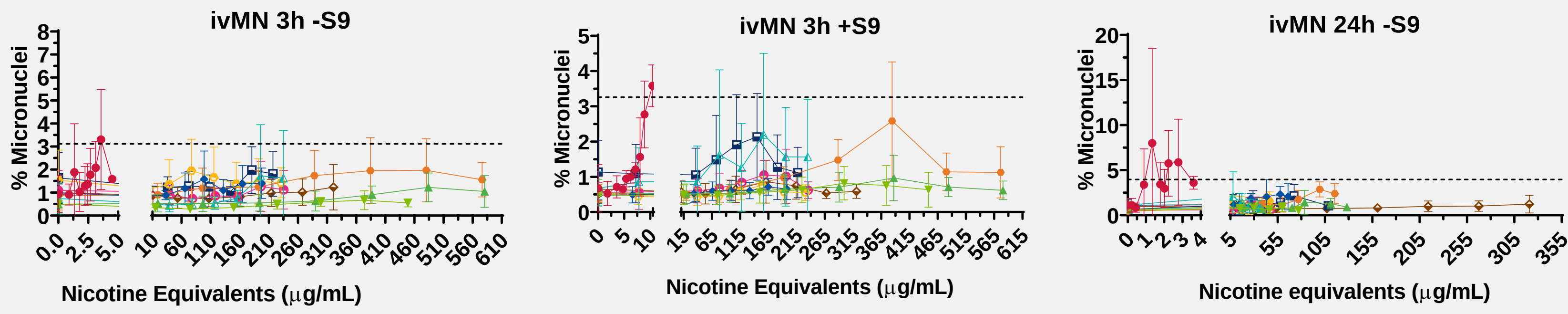


Figure 2. V79 Cell Line ivMN Results by Flow Cytometry. Genotoxic responses were observed for the Combustible but not any of the MO test articles in all three exposure conditions. TPM+GVP test sample (combustible) or CAS extracts exposures were conducted in alignment with the principles of OECD 487 and HC T-503. V79 cells were exposed to the test sample in one of three conditions: $\pm$ S9 with short 3h exposure and 21h recovery or a longer 24h exposure (-S9). Results for the IVMN assay are presented as (average) % MN observed following exposure to test sample. Quadruplicate cell cultures per concentration, n=3, results are mean $\pm$ SD. Positive criteria: Concentration related increase in MN frequency; Statistical increase in MN at one or more concentrations; MN incidence in all replicates exceeds historical range at a concentration (indicated by dotted line).

Results (cont.)

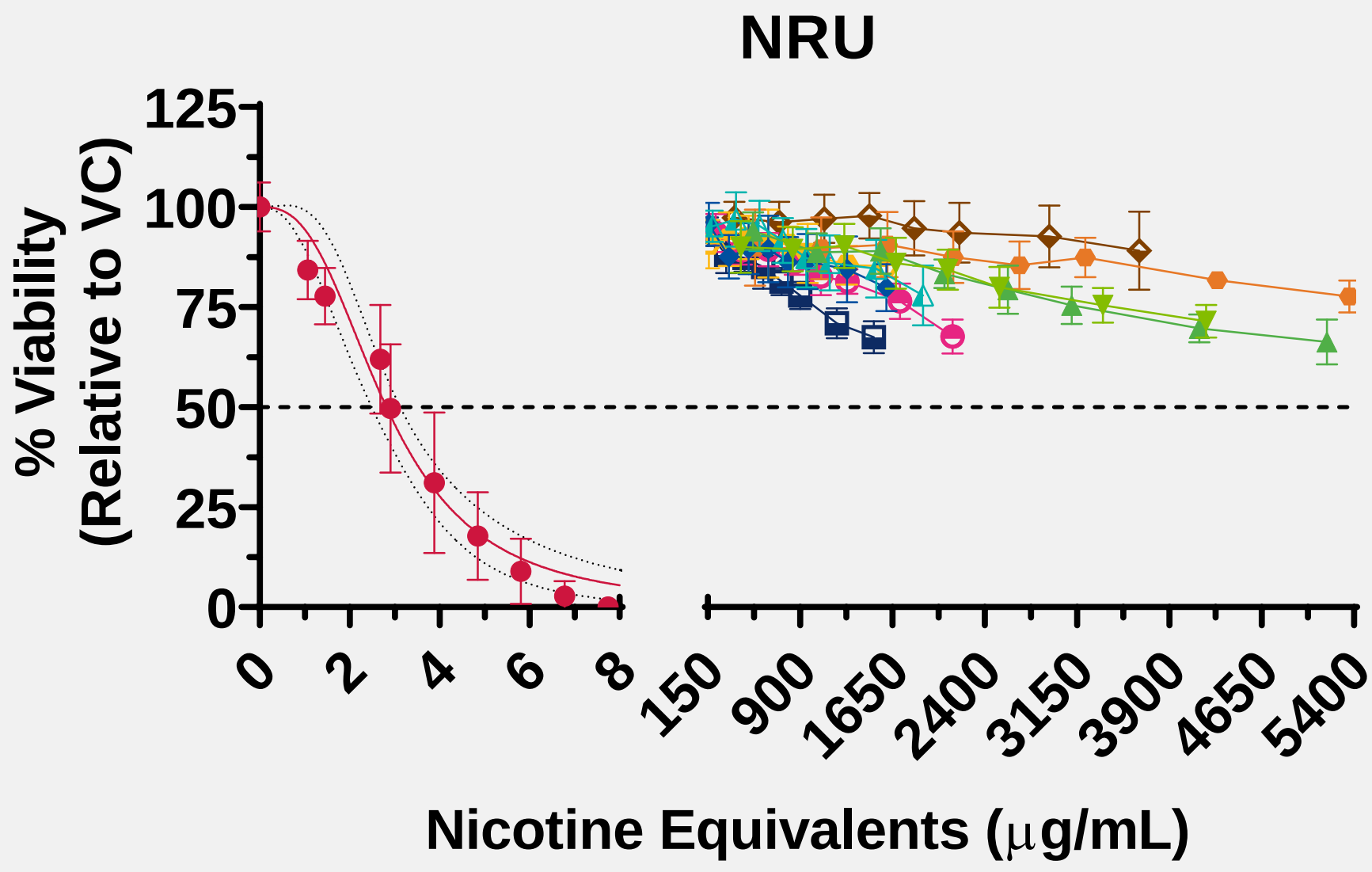


Figure 3. BALB/c 3T3 Cell Line NRU Results. Cytotoxicity was observed for the combustible and no cytotoxicity for any of the MO test articles. NRU results for all test items in both the TPM+GVP and CAS extracts. Combustible cigarette TPM+GVP exposures resulted in cytotoxic responses and calculated IC₅₀ value of 2.8 μ g/mL nicotine equivalents. No indication of cytotoxicity was observed for MO test articles to the maximum concentration of the CAS extract. Six cell cultures per concentration, n=3. Dotted line indicates 50% viability (relative to vehicle control)

Summary & Conclusions

- MO results demonstrated no mutagenicity, genotoxicity, or cytotoxicity across nicotine levels and multiple flavor variants. These results were confirmed with the positive results from the combustible cigarette.
- Combustible cigarette TPM+GVP was found to be mutagenic in strains TA98 ($\pm$ S9), TA100 (-S9), and TA1537 (+S9). MO test articles were not mutagenic in any tested strain (Figure 1).
- Genotoxicity (micronuclei induction) was seen for the combustible cigarette in two of the exposure conditions (3h -S9, 21 h recovery and 24h -S9) and not for any of the MO test articles in any of the exposure conditions (Figure 2).
- Combustible cigarette induced cytotoxicity (IC₅₀ = 2.8 μ g/mL nicotine equivalents) with no cytotoxicity in the MO test articles (i.e., no IC₅₀) in the NRU assay (Figure 3).
- These results add to the growing body of evidence on MO products and support that the use of MO products would reduce consumer exposure to toxic compounds present in CC and provide a potentially lower risk alternative.

