# **Toxicological Assessment of Oral Nicotine Pouch Products Relative to Combustible Cigarettes and Smokeless Tobacco Products Using Regulatory** In Vitro Cytotoxicity and Genotoxicity Assays

## Abstract

Oral nicotine pouches (NPs) are tobacco leaf-free products and therefore contain none or substantially lower levels of the harmful and potentially harmful constituents found in tobacco and tobacco smoke. Thus, NPs may present a potential reduced risk alternative to cigarettes. The goal of this study was to perform toxicological assessment of 12 test NPs (four on!<sup>®</sup> nicotine pouch products [Original and three flavors], each at three nicotine levels [2 mg, 4 mg, and 8 mg]) and compare results to combustible reference cigarettes (3R4F, 1R6F) as well as smokeless tobacco products (reference moist snuff [CRP2.1], reference snus [CRP1.1], four market snus products and six market NPs, using a regulatory in vitro assay battery (MTT for cytotoxicity, Ames for mutagenicity, and micronucleus [MN] for genotoxicity). Cigarette smoke (CS) condensates were collected in ethanol, using ISO intense puffing regimen. All oral products (NPs and smokeless tobacco products, including snus) were extracted in enzyme-free artificial saliva (10% w/v). In the MTT assay, CS was cytotoxic (IC<sub>50</sub> of < 5 µg/mL nicotine), while all tested oral products were non-cytotoxic, even when tested at >10-fold higher nicotine concentrations. In the Ames assay, CS was mutagenic (strain TA98+S9, TA1537+S9), while all oral products were non-mutagenic even when tested at >50-fold higher nicotine concentrations. In the MN assay, CS was genotoxic at ≥1-2 µg/mL nicotine. The CRP1.1, market snus, five market NPs, and five test NPs were non-genotoxic under the testing conditions. The CRP2.1, and some market and test NPs were positive or equivocal for genotoxicity but only at substantially high concentrations (e.g., above the OECD-recommended limit of 5 mg/mL mass for noncytotoxic mixtures) that were at >20-fold higher nicotine concentrations in comparison to CS. In summary, tested NPs and all oral products exhibit substantially lower toxicity profiles, supporting their reduced risk potential compared to combustible cigarettes.

## Introduction

Oral nicotine pouches (NPs) are emerging tobacco-leaf-free innovative products with the potential for tobacco harm reduction (THR). Because these products are tobaccoand smoke-free, they have no or significantly reduced tobacco smoke-related toxicants. They contain tobacco-derived nicotine and various flavor ingredients that are generally recognized as safe (GRAS) for oral use. However, limited data exists regarding their toxicity profile and their position in THR in comparison to combustible cigarettes. In this study, we evaluated the cytotoxic and genotoxic potential of 12 Test NPs using a battery of regulatory in vitro assays (MTT assay, Ames assay, and in vitro MN assay) and their relative toxicity to the combustible and oral tobacco comparators (smokeless tobacco, snus products, and market NPs).

## Materials and Methods



<sup>a</sup> The extracts were diluted in AS for a final maximum concentration of 20% (v/v) for MTT and MN assay and 200  $\mu$ L/plate for Ames assay. <sup>b</sup> For Ames and MN: The response was considered "**Negative**", if none of the criteria for positive responses are fulfilled. The response was considered "**Equivocal**", if the response could not be characterized as either clearly negative or positive, further investigations such as repeating experiments (under different conditions) were conducted.

### Strengths & Limitations

Strengths: 1) We used standardized in vitro cytotoxicity and genotoxicity assays to characterize the novel NP products over the wide dose-response range under the testing conditions. 2) The in vitro toxicity outcomes provide the biological plausibility of reduced-risk potential of smoke-free oral tobacco products, demonstrating clear reduction in toxicity potential compared to combustible cigarettes and comparable biological activity to ST products including snus and market NPs.

Limitations: 1) We used nicotine to represent the extraction efficiency of test materials: based on nicotine analysis, the extraction efficiency for other ingredients (e.g., flavors) are assumed to be proportional but not confirmed analytically. 2) For the MN genotoxicity assay, we used the OECD-recommended limit (5 mg/mL mass) for non-cytotoxic mixtures based on the product weight as mass. 3) We selected test and market NPs based on comparable flavor descriptions; however, they may not represent all market NPs and the use of the results for categorical evaluation warrants caution. 4) Many GRAS food flavors are known to elicit positive response in in vitro genotoxicity assay but are non-genotoxic in vivo. Since NPs contain mixtures of flavors, it is unknown whether the in vitro positive responses seen in MN assay with some NPs are driven by these ingredients with known in vitro genotoxic flags without in vivo genotoxic concerns.

## Results







- concentrations in comparison to CS; see **Table 1** for details)

In vitro toxicological responses of the Test NPs were non-mutagenic, non-cytotoxic, and substantially less genotoxic in comparison to cigarette smoke; in addition, the Test NPs were overall similar in vitro responses compared to oral ST and market NPs, supporting their role in tobacco harm reduction.

**Figure 3.** In vitro MN Genotoxicity Assay (27h-S9-Most Responsive Group): Normalized to Nicotine [A] and to Test Material Mass [B].

• CS condensates, CRP 2.1, and NP-3 were genotoxic. CRP 1.1, four market snus, and five market NPs were non-genotoxic under tested condition

Test NPs showed varying genotoxic responses, sometimes showing genotoxicity at lower tested nicotine concentrations (compared to ST products, see Figure 3A)

Some Test NPs (on!) were genotoxic (equivocal or positive under the testing conditions) but only at substantially higher concentrations (however, at least >90-fold higher nicotine

CS condensates were positive in genotoxicity at substantially lower concentrations compared to all oral ST and NPs. On a mass basis, any positive or equivocal MN responses of Test or market NPs were limited to the high exposure concentrations (> the OECD<sup>4</sup> limit set for testing complex non-cytotoxic mixtures)

### Mariana T. Farcas, Doshi Utkarsh, Mingda Zhang, Yevgeniya V. Prepelitskaya, K. Monica Lee Altria Client Services LLC, Richmond, VA 23219 Center for Research and Technology Society of Toxicology 62<sup>nd</sup> Annual Meeting and ToxExpo March 19-23, 2023 Poster Board number: P580 Abstract ID#: 3456

| Test Product                    | Legends      | Flavor and/or<br>Nicotine strength | Weight<br>Pouch<br>(g) | Cytotoxicity <sup>a</sup><br>IC₅₀      | Ames (Mutagenicity)<br>Strains and Lowest Effective<br>Concentration | In vitro MN<br>(Genotoxicity) <sup>b</sup><br>Lowest Effective<br>Concentration |
|---------------------------------|--------------|------------------------------------|------------------------|--|--|---|
| 3R4F (reference)                | <b></b>      | NA                                 | NA                     | Positive<br>4.27 ± 0.22 µg/mL nicotine | Positive<br>TA98 +S9 & TA1537+S9,<br>2.3 µg nicotine/plate           | Positive*<br>1.44 µg/mL nicotine  |
| 1R6F (reference)                | •            | NA                                 | NA                     | Positive<br>3.82 ± 0.22 µg/mL nicotine | Positive<br>TA98+S9 & TA1537+S9,<br>1.95 µg nicotine/plate           | Positive*<br>0.48 µg/mL nicotine  |
| CRP 1.1 (reference snus)        | ···•         | NA                                 | NA                     | Negative                               | Negative   | Negative  |
| CRP 2.1 (reference moist snuff) |              | NA                                 | 1.00                   | Negative                               | Negative   | Positive <sup>#</sup><br>132.02 µg/mL nicotine                                  |
| Market snus-1                   | _ <b>—</b> — | No flavor, 8.5 mg                  | 1.00                   | Negative                               | Negative   | Negative  |
| Market snus-2                   |              | Mint, 8 mg                         | 1.00                   | Negative                               | Negative   | Negative  |
| Market snus-3                   | <b>_</b>     | White, 8 mg                        | 1.00                   | Negative                               | Negative   | Negative  |
| Market snus-4                   | <b></b>      | Wintergreen, 8 mg                  | 1.00                   | Negative                               | Negative   | Negative  |
| Market NP-1                     | <b>*</b>     | Smooth, 3 mg                       | 0.40                   | Negative                               | Negative   | Negative  |
| Market NP-2                     |              | Mint, 6 mg                         | 0.40                   | Negative                               | Negative   | Negative  |
| Market NP-3                     |              | Peppermint, 6 mg                   | 0.745                  | Positive                               | Negative   | Positive <sup>#</sup><br>31.55 µg/mL nicotine                                   |
| Market NP-4                     | <b>_</b>     | Wintergreen, 4 mg                  | 0.375                  | Negative                               | Negative   | Negative  |
| Market NP-5                     | -            | Mint, 2 mg                         | 0.220                  | Negative                               | Negative   | Negative  |
| Market NP-6                     | -•           | Citrus 2 mg                        | 0.220                  | Negative                               | Negative   | Negative  |
| on! Test NP                     | <b>•</b>     | No flavor, 2 mg                    | 0.25                   | Negative                               | Negative   | Negative  |
|                                 | <b>_</b>     | No flavor, 4 mg                    | 0.25                   | Negative                               | Negative   | Negative  |
|                                 |              | No flavor, 8 mg                    | 0.25                   | Negative                               | Negative   | Positive <sup>#</sup><br>308 µg/mL nicotine                                     |
|                                 | _•_          | Mint, 2 mg                         | 0.25                   | Negative                               | Negative   | Equivocal <sup>#</sup><br>134 µg/mL nicotine                                    |
|                                 |              | Mint, 4 mg                         | 0.25                   | Negative                               | Negative   | Equivocal #<br>210 µg/mL nicotine   |
|                                 |              | Mint, 8 mg                         | 0.25                   | Negative                               | Negative   | Equivocal <sup>#</sup><br>334 µg/mL nicotine                                    |
|                                 | <b>_</b>     | Citrus, 2 mg                       | 0.25                   | Negative                               | Negative   | Positive <sup>#</sup><br>87 µg/mL nicotine                                      |
|                                 | _ <b>_</b>   | Citrus, 4 mg                       | 0.25                   | Negative                               | Negative   | Negative  |
|                                 | -            | Citrus, 8 mg                       | 0.25                   | Negative                               | Negative   | Equivocal #<br>239 µg/mL nicotine   |
|                                 |              | Wintergreen, 2 mg                  | 0.25                   | Negative                               | Negative   | Negative  |
|                                 | <b>——</b>    | Wintergreen, 4 mg                  | 0.25                   | Negative                               | Negative   | Negative  |
|                                 |              | Wintergreen, 8 mg                  | 0.25                   | Negative                               | Negative   | Equivocal #<br>329 µg/mL nicotine   |

<sup>a</sup> Positive indicates <70% relative viability; IC<sub>50</sub> not reported if viability is between 50% and 70%.

<sup>b</sup> Lowest concentration tested with less than 60% cytotoxicity (relative population doubling) that showed %MN response outside of historical range for vehicle control. The data shown in the table are from 27h-S9 treatment, the most responsive group compared to  $4h \pm S9$  treatments. \* Indicates Positive in *in vitro* MN genotoxicity assay at concentrations > 5 mg/ml mass (the OECD<sup>4</sup> limit set for testing complex non-cytotoxic mixtures)

# Indicates Positive or Equivalent in *in vitro* MN genotoxicity assay at concentrations < 5 mg/ml mass (the OECD<sup>4</sup> limit set for testing complex non-cytotoxic mixtures)

### References

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• The test materials were prepared at Enthalpy Analytical, and the *in vitro* assays were conducted at Charles River Laboratories