Comparative Toxicity Assessment of Oral Nicotine Pouches to Combustible Cigarettes, Smokeless Tobacco Products and Market Nicotine Pouches Using Regulatory in vitro Cytotoxicity, Mutagenicity, and Genotoxicity Assays

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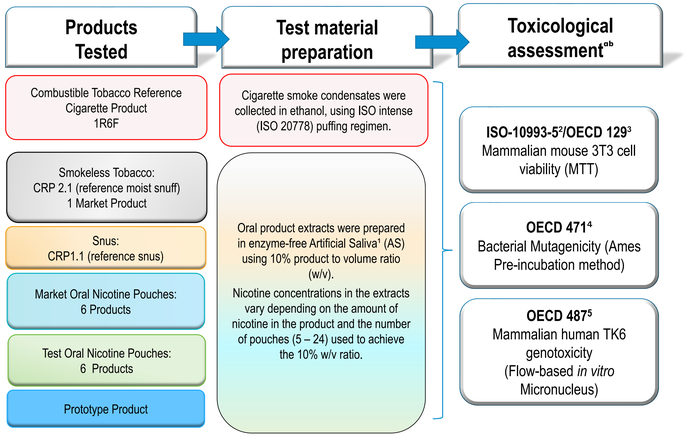
Background and Purpose

Oral nicotine pouches (NPs) are tobacco leaf-free products and therefore contain substantially lower levels of the harmful and potentially harmful constituents found in cigarette smoke and oral products containing tobacco (e.g., moist snuff and snus). The goal of this study was to perform an in vitro toxicological assessment of six Test NPs (mint, wintergreen, and tobacco-flavored products, each at two nicotine levels [6 mg and 12 mg]) and compare the results to a combustible reference cigarette (1R6F), and oral products (reference moist snuff [CRP2.1], reference snus [CRP1.1], one market moist snuff and seven market NPs). For the Test mint NP, one prototype NP was also tested to investigate the role of key ingredients (maltols) in the in vitro micronucleus (MN) outcomes, in addition to the in vivo genotoxicity follow-up study (presented separately by Zhang et al, poster P212).

Materials and Methods

***IN VITRO* TOXICOLOGICAL ASSESSMENT WORKFLOW**



[a] The test articles were diluted in AS for a final maximum concentration of 20% (v/v) for MTT and MN assay and 200 µL/plate for Ames assay.
[b] Criteria for evaluating assay responses was based on respective guidelines: ISO10993-5, OECD TG129 (cytotoxicity); OECD TG471 (Ames) and OECD TG487 (MN). For Equivocal outcomes, where the response could not be characterized as either clearly negative or positive, further investigations such as repeating experiments (under different conditions) were conducted.

Conclusions

- *Test NPs were non-mutagenic and non-cytotoxic. Some Test NPs showed genotoxicity but only at the high exposure (above the OECD recommended 5 mg mass/mL limit for non-cytotoxic mixtures)*
- *Test NPs were consistently and substantially less toxic than cigarette smoke and overall comparable to ST and select market NPs*
- *For Test Mint NPs, the in vitro genotoxicity was deemed to be associated with key ingredients with known in vitro positive results but without in vivo sequelae (confirmed by a follow-up in vivo genotoxicity study)*
- *Overall, these results support the reduced risk potential of the Test NPs and its role in tobacco harm reduction*

Results

Figure 1. MTT Cytotoxicity Assay.

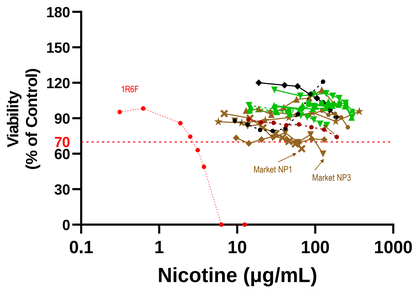


Figure 2. Ames Mutagenicity Assay (TA98+S9).

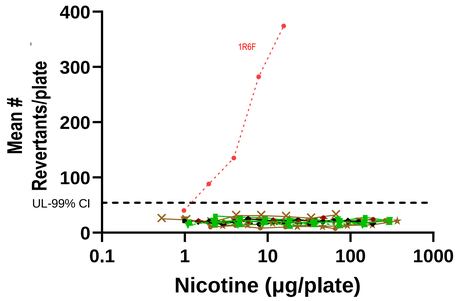
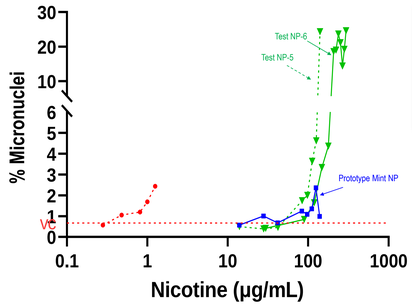


Figure 4. *In vitro* Genotoxicity Assay of Test Mint and Prototype NP.



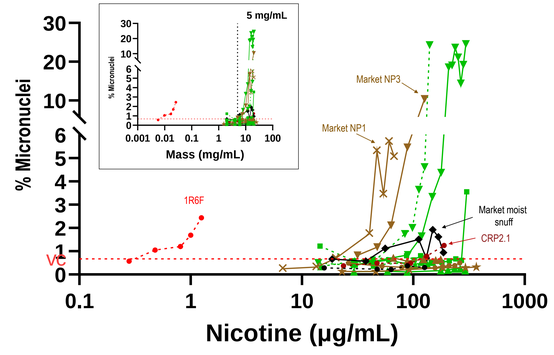
Cytotoxicity
- 1R6F was cytotoxic (IC_{50} < 5 µg/mL nicotine)
- All Test NPs were non-cytotoxic
- Most tested oral comparator products, were non-cytotoxic
- Two Market NPs (NP1, NP3) showed minimal toxicity, with viability of 60-64% at the highest nicotine concentration

Mutagenicity
- 1R6F was mutagenic (only strains TA98+S9 and TA1537+S9)
- All oral products, including Test NPs were non-mutagenic in all strains, even when tested at 60-170-fold higher nicotine concentrations compared to 1R6F

- Comparison testing of the prototype product (same as Test Mint but without maltols) showed substantially reduced genotoxicity response compared to Test Mint NP, demonstrating that in vitro MN response of Test Mint NP is primarily driven by maltols

Supporting Tox information:
- Toxicological ingredient assessment shows that maltol levels in Test Mint NP does not have in vivo toxicological or carcinogenic concerns (see **Poster P137**)
- Test Mint NP were tested under ICH guidance for in vivo MN and DNA damage: the genotoxicity outcomes confirm negative in vivo genotoxicity potential (see **Poster P212**)

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



Genotoxicity
- 1R6F was genotoxic at ≥0.5 µg nicotine/mL
- The CRP 2.1 and market moist snuff were also positive for genotoxicity but only at >100-fold higher nicotine concentrations than 1R6F
- CRP 1.1 snus was non-genotoxic
- Test Wintergreen (6 mg & 12 mg) and Tobacco (6mg) NPs were non-genotoxic at the concentrations tested
- Test Tobacco (12 mg) and Mint (6 & 12 mg) Test NPs were positive in MN; however, at >100-fold higher nicotine concentrations than 1R6F; were within the range of responses from tested oral products (ST and Market NPs) and at the high exposure concentrations above OECD[4] limit (5 mg/mL mass for non-cytotoxic mixtures (see inset)
- MN response of Test Mint NP was further evaluated against the response of the prototype NP (Fig 4.)

Table 1. Product Information, Legends, and Summary of *in vitro* Toxicity Assessment.

Test Product	Legends	Flavor, nicotine strength	Cytotoxicity IC_{50}	Ames (Mutagenicity) Strains and Lowest effective concentration	*In vitro* MN (Genotoxicity) [b] Lowest effective concentration
1R6F (reference)	- -●- -	NA	**Positive** 3.82 ± 0.22 µg nicotine/mL	**Positive** TA98+S9 & TA1537+S9, 1.95 µg nicotine/plate	**Positive** 1 µg nicotine/mL
CRP 1.1 (reference snus)	- -●- -	NA	Negative	Negative	Negative
CRP 2.1 (reference moist snuff)	- -●- -	NA	Negative	Negative	**Positive** 188 µg nicotine/mL
Market moist snuff	—◆—	Wintergreen, NA	Negative	Negative	**Positive** 112 µg nicotine/mL
Market NP-1	—×—	Wintergreen, 6 mg	**Positive**[a]	Negative	**Positive** 40 µg nicotine/mL
Market NP-2	—▲—	Wintergreen, 4 mg	Negative	Negative	Negative
Market NP-3	—▼—	Peppermint, 6 mg	**Positive**[a]	Negative	**Positive** 63 µg nicotine/mL
Market NP-4	—●—	Chill, 6 mg	Negative	Negative	Negative
Market NP-5	—★—	Cool Mint, 6 mg	Negative	Negative	Negative
Test NP-1	- -▲- -	Wintergreen, 6 mg	Negative	Negative	Negative
Test NP-2	—▲—	Wintergreen, 12 mg	Negative	Negative	Negative
Test NP-3	- -■- -	Tobacco, 6 mg	Negative	Negative	Negative
Test NP-4	—■—	Tobacco, 12 mg	Negative	Negative	**Positive** 301 µg nicotine/mL
Test NP-5	- -▼- -	Mint, 6 mg	Negative	Negative	**Positive** 84 µg nicotine/mL
Prototype Test NP	—■—	Mint, 6 mg	Negative	Negative	**Positive** 111 µg nicotine/mL
Test NP-6	—▼—	Mint, 12 mg	Negative	Negative	**Positive** 119 µg nicotine/mL

[a] Positive indicates <70% relative viability; IC_{50} not reported if viability is between 50% and 70%.
[b] Lowest concentration tested with less than 60% cytotoxicity (relative population doubling) showing statistically significant increase in the percent of micronucleated. cells relative to concurrent vehicle control.
The data shown in the table are from 27h-S9 treatment, the most responsive group compared to 4h ± S9 treatments.

Strengths & Limitations

Strengths: 1) We used standardized in vitro cytotoxicity and genotoxicity assays to characterize the novel NP products. 2) The in vitro toxicity outcomes support reduced-risk potential of smoke-free oral tobacco products, demonstrating clear reduction in toxicity potential compared to combustible cigarettes. 3) We conducted ingredient-specific in vitro investigation to substantiate that in vitro genotoxicity for Test Mint product was primarily driven by key ingredients (maltols) but without in vivo sequels (Zhang et al., poster P212).
Limitations: 1) We evaluated the test material extraction efficiency based on nicotine only. 2) For the MN genotoxicity assay, we used the OECD-recommended limit (5 mg/mL mass) for non-cytotoxic mixtures: biological significance of genotoxicity above 5 mg/mL is not further evaluated. 3) We selected a variety of tobacco and market NPs with different nicotine levels and flavor varieties at the time of testing. This limited selection of market NPs may not represent the totality of available products but does provide sufficient context by which to compare the market products and the candidate products.

References

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4. OECD. (2020). OECD Guideline for Testing Chemicals Test Guideline 471, Bacterial Reverse Mutation Test.
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7. Pitegoff, M., et al (2024). Hazard Identification and Risk Assessment of Non-Nicotine Ingredients in Oral Nicotine Pouches (3636/P137). 63rd Annual Meeting & ToxExpo. Salt Lake City, Utah. March 10-14, 2024.

Acknowledgments

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