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

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Abstract

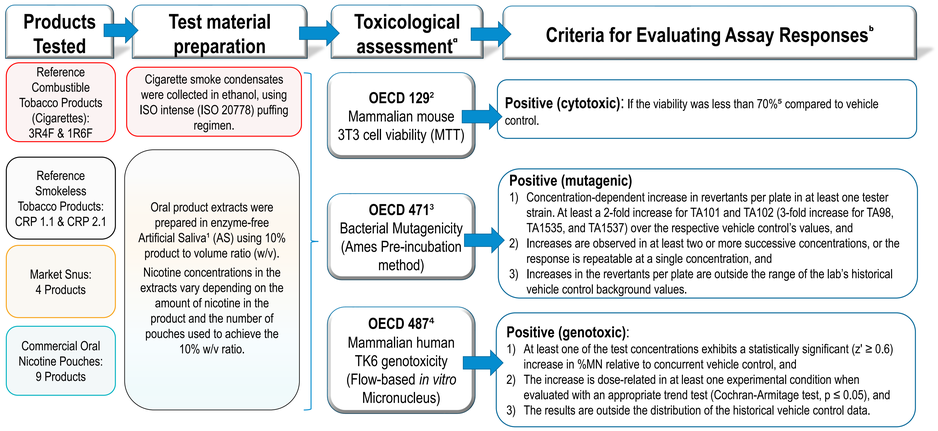
Oral nicotine pouches (NPs) are non-combustible and tobacco leaf-free nicotine products that may fall on the lower end of the risk continuum spectrum with combustible cigarettes at the highest end, holding promise for tobacco harm reduction (THR). The goal of this study was to perform a toxicological assessment of nine market NPs using a regulatory *in vitro* assay battery (MTT assay for cytotoxicity, Ames assay for mutagenicity, and micronucleus [MN] assay for genotoxicity) and compare the results to combustible reference cigarettes (3R4F, 1R6F) and oral tobacco products (reference moist snuff [CRP2.1], reference snus [CRP1.1], and four market snus). Cigarette smoke (CS) condensates were collected in ethanol, using ISO intense puffing regimen, while oral tobacco products, and market NPs were extracted in enzyme-free artificial saliva (10% w/v). In MTT, CS condensate was cytotoxic (IC_{50} of < 5 µg/mL nicotine), while all tested oral tobacco extracts were non-cytotoxic, even when tested at >10-fold higher nicotine concentrations. The Ames assay demonstrated that CS condensates were mutagenic (strain TA98+S9, TA1537+S9), while the oral tobacco extracts were non-mutagenic even when tested at >65-fold higher nicotine concentrations. In the MN assay, CS condensates were genotoxic between 1-2 µg/mL nicotine, while CRP2.1 and three NP extracts were genotoxic only when tested at >90-fold higher nicotine concentrations in comparison to CS condensate. In contrast, the remaining six market NPs, CRP 1.1, and market snus extracts were non-genotoxic even when tested at >250-fold higher nicotine concentrations than CS condensates. In summary, tested NP products and oral tobacco products had substantially lower toxicity profiles than cigarettes, and tested NPs had a comparable toxicity profile to oral tobacco products, supporting their reduced-risk potential in THR.

Introduction

Smoke-free nicotine products, such as oral NPs, are potential reduced-risk alternatives to conventional cigarettes for adult smokers. They do not have tobacco or tobacco-related toxicants but include tobacco-derived nicotine and various flavor ingredients that are designated as GRAS (Generally Recognized as Safe) for oral use. As this product category is relatively new, there is limited data on their toxicity profiles in comparison with combustible cigarettes and their role in THR. In this study, we tested selected commercial NPs using a battery of regulatory *in vitro* assays (MTT assay, Ames assay, and *in vitro* MN assay), to evaluate their cytotoxicity and genotoxicity potential relative to the combustible and oral tobacco comparators (smokeless tobacco and snus products).

Materials and Methods

IN VITRO TOXICOLOGICAL ASSESSMENT WORKFLOW



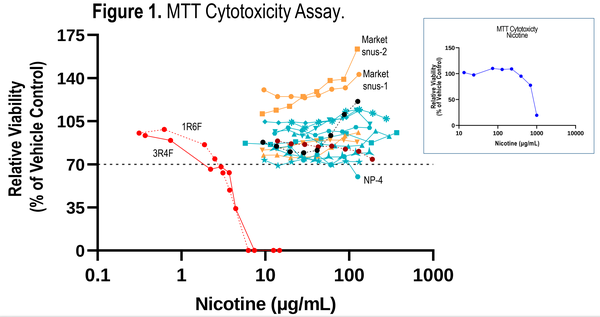
[a] The test articles were diluted in AS for a final maximum concentration of 20% (v/v) for MTT and MN assay and 200 µL/plate for Ames assay.
[b] For Ames and MN: The response was considered **"Negative"**, if none of the criteria for positive responses are fulfilled. The response was considered **"Equivocal"**, 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 OTDN products. 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.
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 complete but not confirmed analytically. 2) For the MN genotoxicity assay, we used the OECD-recommended limit (5 mg/mL) for non-cytotoxic mixtures: biological significance of genotoxicity above 5 mg/mL is not further evaluated. 3) We selected market NPs with different nicotine levels, flavor varieties, and manufacturers, which may be considered a strength, however, this may not be representative of the totality of available 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*. When evaluating the product extracts, 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.

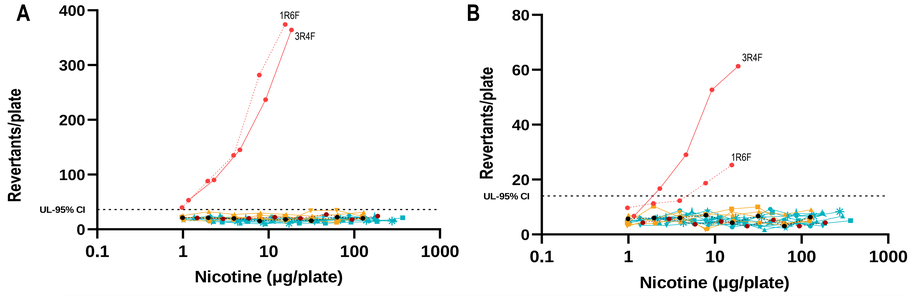
Results

Figure 1. MTT Cytotoxicity Assay.



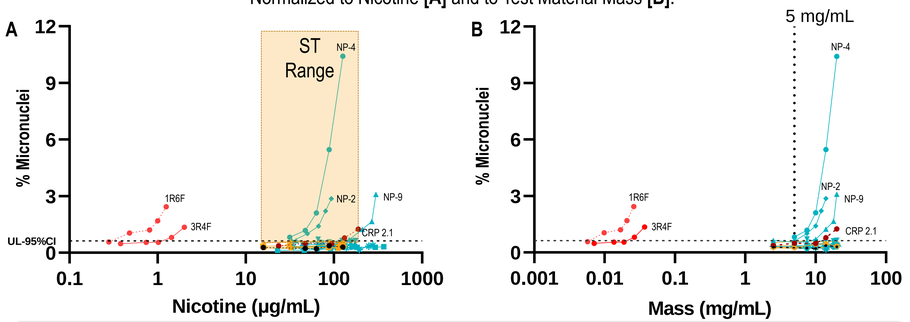
- CS condensates were cytotoxic (IC_{50} of < 5 µg/mL nicotine), while tested oral product extracts were non-cytotoxic, even when tested at >10-fold higher nicotine concentrations
- The increase in viability observed in some oral tobacco products (e.g., market snus products) was not likely driven by nicotine (see **Insert Figure**: MTT cytotoxicity with nicotine only)
- Commercial NP-4 was weakly cytotoxic: viability of 60% at the highest nicotine concentration (>100-fold higher nicotine concentrations compared to CS)

Figure 2. Ames Mutagenicity Assay (TA98+S9 **[A]** and TA1537+S9 **[B]**).



- CS condensates were mutagenic (strain TA98+S9, TA1537+S9), while the oral products were non-mutagenic in all strains even when tested at >65-fold higher nicotine concentrations

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



- CS condensates were genotoxic (1-2 µg/mL nicotine), while CRP 2.1 and three NP extracts were genotoxic only when tested at >90-fold higher nicotine concentrations in comparison to CS condensate. The remaining six market NPs, CRP 1.1, and market snus extracts were non-genotoxic even when tested at >250-fold higher nicotine concentrations than CS condensates
- In comparison to oral tobacco comparators, NPs showed varying genotoxic responses, wherein some showed more, and others showed similar genotoxicity at comparable nicotine concentrations (based on Smokeless Tobacco (ST) Concentration Range box in **Figure 3A**)
- On a mass basis, CS condensates were positive at ~90-fold lower concentrations than the OECD limit of 5 mg/mL, whereas the positive responses for oral products were observed at the exposure concentrations only above this OECD[4] limit set for testing complex non-cytotoxic mixtures
- Since genotoxic responses were independent of nicotine concentrations, it is likely that nicotine is not driving the *in vitro* genotoxic responses at these concentrations

In vitro toxicological responses with NPs varied depending on product type; however, similar to oral tobacco products, the selected commercial NPs were found to be non-mutagenic, non-cytotoxic, and substantially less genotoxic in comparison to mutagenic, cytotoxic, and genotoxic cigarette smoke, supporting their reduced risk potential

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

Test Product	Legends	Flavor and Nicotine strength	Weight Pouch (g)	Cytotoxicity [a] IC_{50}	Ames (Mutagenicity) Strains and Lowest Effective Concentration	*In vitro* MN (Genotoxicity) [b] Lowest Effective Concentration
3R4F (reference)		NA	NA	Positive 4.27 ± 0.22 µg/mL nicotine	Positive TA98 +S9 & TA1537+S9, 2.3 µg nicotine/plate	Positive 1.44 µg/mL nicotine
1R6F (reference)		NA	NA	Positive 3.82 ± 0.22 µg/mL nicotine	Positive TA98+S9 & TA1537+S9, 1.95 µg nicotine/plate	Positive 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 132.02 µg/mL nicotine
Market snus-1		No flavor, 8.5 mg	1.00	Negative	Negative	Negative
Market snus-2		Mint, 8 mg	1.00	Negative	Negative	Negative
Market snus-3		White, 8 mg	1.00	Negative	Negative	Negative
Market snus-4		Wintergreen, 8 mg	1.00	Negative	Negative	Negative
Commercial NP-1		Smooth, 3 mg	0.40	Negative	Negative	Negative
Commercial NP-2		Coffee, 6 mg	0.40	Negative	Negative	Positive 50.10 µg/mL nicotine
Commercial NP-3		Mint, 6 mg	0.40	Negative	Negative	Negative
Commercial NP-4		Peppermint, 6 mg	0.745	Positive	Negative	Positive 31.55 µg/mL nicotine
Commercial NP-5		Wintergreen, 4 mg	0.375	Negative	Negative	Negative
Commercial NP-6		Mint, 2 mg	0.220	Negative	Negative	Negative
Commercial NP-7		Citrus 2 mg	0.220	Negative	Negative	Negative
Commercial NP-8		Dragon Fruit, 7 mg	0.356	Negative	Negative	Negative
Commercial NP-9		Cinnamon, 7 mg	0.374	Negative	Negative	Positive 149.30 µg/mL nicotine

[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) 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 ± S9 treatments.

References

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Acknowledgments

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