Comparative toxicological evaluation of nicotine gum products to combustible cigarette using regulatory in vitro assays.

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Background and Purpose

Regulatory in vitro hazard identification assays are critical tools in assessing toxicological properties of various products, including tobacco derived nicotine-containing products. While extensive research has been conducted on the in vitro toxicity of traditional nicotine delivery systems such as combustible cigarettes, there is a notable gap in the literature regarding the in vitro toxicity of next generation nicotine products such as gums.

This study evaluates in vitro cytotoxicity and genotoxicity of nicotine gum extracts. Nine commercially available nicotine gum products were compared to the reference combustible cigarette 1R6F, using established regulatory in vitro assays. This comparative study aims to provide a better understanding of the reduced risk potential of nicotine gums relative to traditional combustible cigarettes. This work will contribute to the existing body of knowledge and inform regulatory decisions-regarding the toxicological hazard profile of nicotine-containing products.

Materials and Methods

Test Articles: Nine commercial nicotine gum products (MP-1 through MP-9) with nicotine strengths ranging from 2 to 6 mg per piece, were used for in this study. Several mint (MP1, -2, -4, -7 and -8) and fruit (MP-3, -5, -6 and -9) flavors variants were evaluated.

Extract Preparation[1]: Briefly, frozen (-80 ℃) commercial gum products were ground using the Grindomix GM200 (Retsch, 3-cycles alternating hit and cut at 4000 rpm and 7000 rpm, respectively, for 15 seconds each). The ground gum was then added to a suitably sized sealable vessel, and 10mL of artificial saliva was added for each unit of ground gum (1 gum/10mL). The gum solution was vortexed and then shaken on an orbital shaker at 200 rpm for 3 hours. The resulting extract was centrifuged at 4000 rpm, and the supernatant was filtered twice (0.2 µm) for sterility. The final extracts were evaluated for nicotine recovery relative to the product label's nicotine content (Table 1). Additionally, extracts were prepared using assay-specific media (DMEM for cytotoxicity and RPMI for genotoxicity).

Combustible Tobacco Product (1R6F Cigarettes) Collection: Cigarettes were puffed according to ISO intense (ISO 20778[2]) puffing regimen[3] (55 mL puff, 2 sec. puff, 30 sec. interval, 100% vent blocked) on a rotary smoking machine and collected on a conditioned 92 mm Cambridge filter pad (CFP) connected in series with an impinger filled with 30 mL USP-grade ethanol cooled in an ice water bath. The CFP was extracted with impinger contents and then filter-sterilized to produce the whole smoke condensate.

In Vitro Assessment:

Genotoxicity (Micronucleus-MN): OECD 487 Mammalian human TK6 micronucleus assay: Flow-based analysis, 4h±S9 and 22h-S9. Doses evaluated were 0.5, 1.0, 5.0, 10, 30, 40 and 80% v/v of a 1gum/10mL extract. 5000 nuclei events counted per culture with 4 replicates per dose. Cytotoxicity determined by Relative Increased Cell Counts (RICC)

Cytotoxicity (Neutral Red Uptake-NRU): ISO-10993-5/OECD 129 Mammalian mouse 3T3 cell viability: 48h treatment. Doses were 1.25, 2.5, 5.0, 10, 20, 40 and 80% v/v of a 1gum/10mL extract. Cytotoxicity was defined as viability <70%.

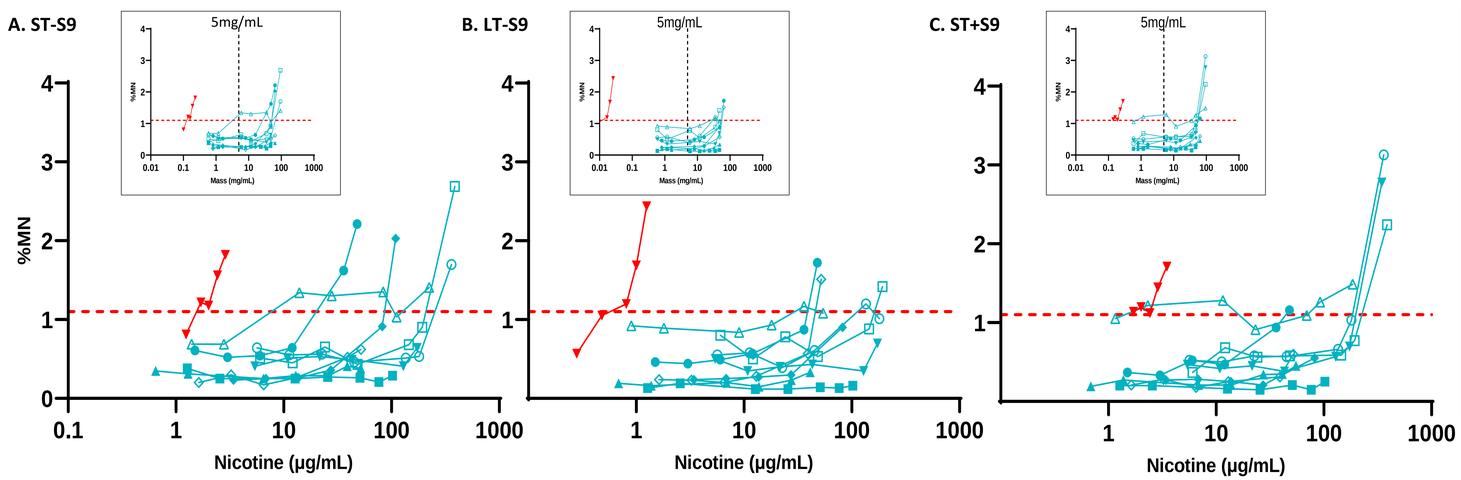
pH and Osmolality: In both assays: pH was evaluated and found comparable to media controls. Osmolality was limited to < 550 mOsm/kg (equivalent to 1% DMSO measurement).

References

[1] Hurtado, SB. et al., Optimization of Extraction and In Vitro Evaluation of Market Nicotine Gums, TSRC meeting, September 8-11, 2024, Atlanta GA, USA
[2] ISO 20778 (2018). Cigarettes - Routine analytical cigarette smoking machine - Definitions and standard conditions with an intense smoking regime.
[3] Doshi, U. et al., Comparison of in vitro Cytotoxicity and Genotoxicity of Condensates Derived from E-vapor Products and Combustible Cigarette, SOT Meeting, March 11-15, 2018, San Antonio TX, USA

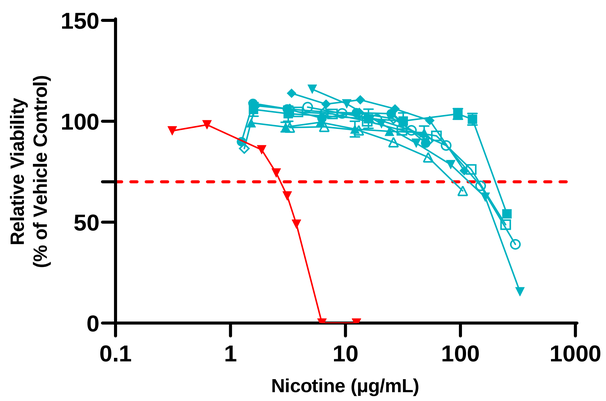
Results

Figure 1. In vitro Micronucleus Assay. Gum extracts were prepared directly into RPMI for application up to 80% v/v to the cells. Osmolality limited evaluated doses to 40% v/v or less. Cytotoxicity was evaluated using relative increase in cell counts (RICC). Data normalized to nicotine and mass (inset) shown for A. 4-hour without metabolic activation (ST-S9) B. 22-hour without metabolic activation (LT-S9) and C. 4-hour with metabolic activation (ST+S9).



Genotoxicity
- 1R6F is genotoxic at <0.5 µg/mL nicotine.
- Gum extracts were evaluated at nicotine concentrations 16- to 53-fold higher than 1R6F.
- 6 of 9 nicotine gum products tested were positive for genotoxicity.
  - Responding gum nicotine concentrations were >24-fold above those of 1R6F.
  - Statistically significant. positive responses observed at > 5 mg/mL mass only.
- Responses were independent of nicotine concentration.

Figure 2. Neutral Red Uptake Assay. Gum extracts were prepared directly into DMEM for application up to 80% v/v to the cells. Osmolality limited to doses of 550 mOsm/kg or less. Relative viability in in BALB/c 3T3 cells was normalized to nicotine.



Cytotoxicity
- 1R6F was cytotoxic (IC_{50} = 3.82 µg/mL nicotine).
- Gum extracts were evaluated at nicotine concentrations up to 66.8-fold higher than responding 1R6F concentrations.
- Five nicotine gum extracts exhibited cytotoxicity.
  - All cytotoxic responses were from the same brand (Brand 2, see Table 1).
- Responding gum concentrations were >18-fold above those of 1R6F.
- Responses were independent of nicotine concentration.

Table 1. Summary of Nicotine Recovery, Cytotoxicity and Genotoxicity Responses.

Product	Nicotine (mg/piece)	Nicotine Recovery (%)	Cytotoxicity	Genotoxicity
MP-1	4	80	Yes	No
MP-2	2	65	No	No
MP-3	4	70	Yes	Yes
MP-4	6	70	Yes	Yes
MP-5	6	70	Yes	Yes
MP-6	6	66	Yes	Yes
MP-7	2	61	No	Yes
MP-8	4	68	No	Yes
MP-9	2	66	No	No

These results suggest substantially lower biological activity of the nicotine gum products relative to combustible cigarettes.

Conclusion

- The selected preparation method for gum products showed nicotine recovery in the range of 61% to 80%, suggesting the method is fit for purpose across multiple gum products.
  - The method allowed for testing of ≥10-fold nicotine concentrations compared to cigarette and exceeded the OECD guidance recommendations for mixtures demonstrating both it's appropriateness and effectiveness.
- The reference cigarette 1R6F showed cytotoxicity and genotoxicity.
- While some gum products showed cytotoxicity and genotoxicity, the biological responses were only observed at several-fold higher nicotine concentrations compared to reference cigarette.
  - Cytotoxicity was observed in products where the dose evaluated was less limited by osmolality.
  - Gum genotoxic responses observed were at concentrations > 5mg/mL mass.
- Both cytotoxicity and genotoxicity from nicotine gums were independent of nicotine strength, likely driven by non-nicotine ingredients.

Acknowledgements

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2. Collection of 1R6F cigarette condensate; extraction, nicotine assessment and in vitro testing of gum products conducted at McKinney Specialty Labs, Richmond, VA.

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