Evaluation of Genotoxicity of Cinnamon-flavored Nicotine Pouch Products Using In Vitro New Approach Methodologies

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New approach methodologies provide valuable mechanistic insights into the genotoxicity hazard of nicotine pouch products

INTRODUCTION

- Oral nicotine pouch (NP) products represent a class of reduced-risk products whose toxicological assessment presents unique challenges.
- This study addresses a key discrepancy in toxicological evaluation of NP products containing GRAS (generally recognized as safe) ingredients that trigger a positive in vitro micronucleus (MN) response but have no corresponding in vivo effects.
- We used an in vitro new approach methodology, the ToxTracker assay, which was recently accepted into the OECD test guidelines programme, to investigate the specific mechanistic pathways behind these results, using commercially available cinnamon-flavored NP products as an example, to provide context for in vitro positive MN results.

METHODS

- **Test Articles:** Three commercially available cinnamon flavored oral NP products from 3 different manufacturers: NP-1 (6 mg nicotine), NP-2 (3 mg nicotine) and NP-3 (7 mg nicotine).
- **Preparation of Test Materials for in vitro testing and analytical characterization:** The NP products (including pouch material) were extracted in artificial saliva buffer at product to solvent ratio of 10% (w/v), shaken for 2h at 37˚C and centrifuged. After centrifugation, the material was sterile-filtered through 0.2 µm filter and stored frozen until further use.
- **Analytical Characterization of Test Materials:** The test materials (NP product extracts) were analyzed for nicotine and cinnamaldehyde content using gas chromatography-mass spectroscopy (GC-MS).
- **In Vitro Mutagenicity & Genotoxicity Assay:** The test materials were subjected to bacterial reverse mutation (Ames) assay in 5 *Salmonella typhimurium* strains (TA98, TA100, TA102, TA1535, TA1537) ± metabolic activation (S9) and to in vitro micronucleus assay in human TK6 cells (short term (ST) ±S9 and long term (LT) -S9) according to the OECD test guidances 471[1] and 487[2], respectively.
- **ToxTracker GFP-reporter Assay[3]:** Six mouse embryonic cell lines containing different fluorescent reporters (green fluorescent protein (GFP) signals) were exposed to test materials in the presence and absence of 0.4% phenobarbital-naphthoflavone-induced rat liver S9 (S9) for 24h. Biomarker expression was analyzed 24h after exposure initiation and GFP expression and relative survival were measured using flow cytometry. Aneugenicity was assessed by cell-cycle analysis. To assess indirect genotoxicity due to oxidative stress, the ToxTracker assay was performed in the presence of 10 mM reactive oxidative species (ROS) scavengers (n-acetyl cysteine (NAC) or glutathione (GSH)). Information on biomarkers and the type of damage they detect can be seen in Table 1. The ToxTracker assay is considered to have a positive response when a compound induces ≥2-fold increase in GFP expression for any of the reporter cell line end points. Only GFP inductions occurring at test material concentrations manifesting ≤75% cytotoxicity were used for the ToxTracker analysis.

Table 1: Specificity of the ToxTracker Reporters

Damage Type	Biomarker Gene	Mechanism of Action
DNA damage	Bscl2	Mutagenic DNA lesions
	Rtkn	DNA double-strand breaks
Oxidative Stress	Srxn1	ROS production-Nrf2 dependent
	Blvrb	ROS production-Nrf2 independent
Protein Damage	Ddit3	Unfolded protein response
Cell Stress	Btg2	p53 signaling

Details of study conduct: Preparation of test materials and analysis of nicotine in the extracts were conducted at McKinney Specialty Labs, Richmond, VA; analytical determination of cinnamaldehyde in the extracts was conducted at ALCS, Richmond VA; ToxTracker based assessment was conducted at Toxys Inc, The Netherlands.

RESULTS

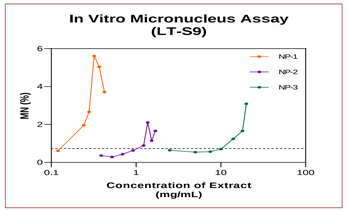
In Vitro Mutagenicity & Genotoxicity Assay

Table 2: Summary of test materials and their mutagenicity and genotoxicity outcomes from in vitro regulatory assays

Test Article	Nicotine Strength	Nicotine in extract (mg/mL)	Metabolic Activation	Ames Assay	Micronucleus (MN) Assay	
					ST	LT
NP-1	6 mg	0.81	-S9	Negative	Positive	Positive
			+S9	Negative	Positive	NA
NP-2	3 mg	0.69	-S9	Negative	Positive	Positive
			+S9	Negative	Positive	NA
NP-3	7 mg	1.44	-S9	Negative	Negative	Positive
			+S9	Negative	Negative	NA

ST: Short term; LT: Long term; S9: metabolic activation, NA=Not applicable

Figure 1: Representative concentration-responses of cinnamon flavored nicotine pouch extracts in the MN assay (long term -S9 group).

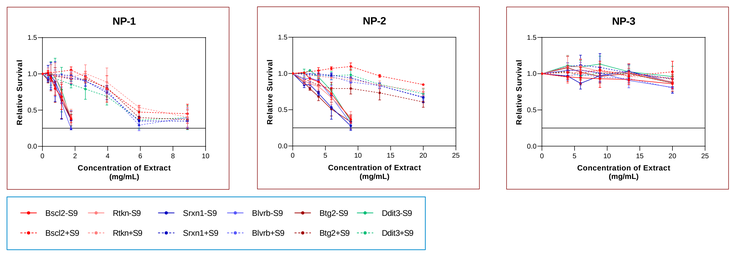


--------: 95% Upper limit of the confidence Interval of Lab's historical range for vehicle control

- The cinnamon-flavored nicotine pouches were negative in the Ames assay.
- All 3 nicotine pouch products were genotoxic in the in vitro micronucleus (MN) assay. Based on concentrations driving the genotoxic response, following rank ordering was observed: NP-1>>NP-2>>NP-3
  - ❖ NP-1 and NP-2 showed genotoxicity across all treatment groups, whereas NP-3 was genotoxic only in the long-term treatment group without S9.
  - ❖ The observed effects were independent of nicotine concentration in the extract.

Cytotoxicity (ToxTracker Assay) & Cinnamaldehyde Levels in the Extracts

Figure 2: Cytotoxicity (Relative survival) curves of nicotine pouch extracts (NP-1 to NP-3) in presence and absence of metabolic activation (S9) in ToxTracker assay. The dark line in the graphs represents 25% viability threshold (75% toxicity)



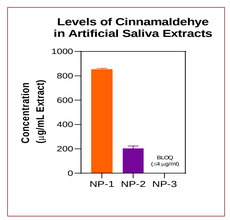
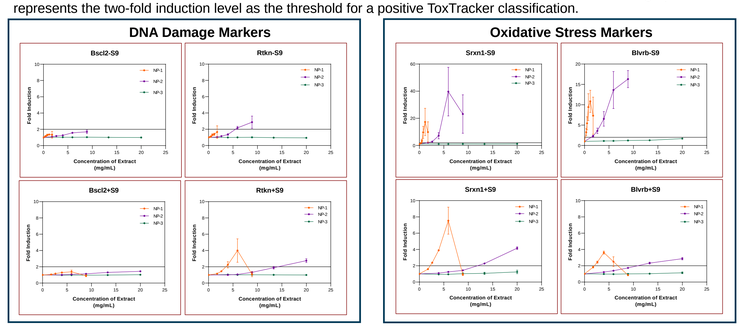


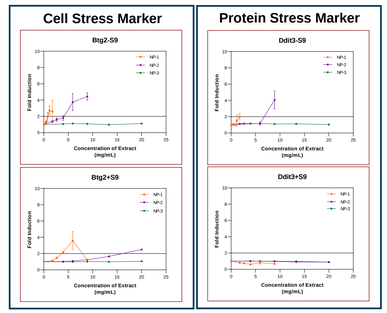
Figure 3: Cinnamaldehyde levels in aqueous nicotine pouch extracts as measured by GC-MS

- Both NP-1 and NP-2 exhibited concentration-dependent cytotoxicity in the ToxTracker assay, while NP-3 was non-cytotoxic.
  - ❖ The cytotoxicity was reduced in the presence of S9.
- Cytotoxicity appeared to correlate with the levels of cinnamaldehyde in the extracts: NP-1 had the highest level of cinnamaldehyde and was most cytotoxic, while NP-3 had undetectable levels and was non-cytotoxic.

ToxTracker Assay

Figure 4: Activation of the six different ToxTracker reporters following exposure to increasing concentrations of the three cinnamon-flavored nicotine pouch extracts in the presence and absence of S9. The dark line in the graphs represents the two-fold induction level as the threshold for a positive ToxTracker classification.





- Exposure to NP-1 induced genotoxicity marker (Rtkn) only in presence of S9 and exposure to NP-2 induced Rtkn, both in the presence and absence of S9.
  - At lower concentrations, activation of oxidative stress markers (Srxn1 and Blvrb) was observed in response to both the extracts and neither extract was aneugenic (from cell cycle analysis, data not shown), collectively suggesting that the observed activation of Rtkn may be indirect and driven by oxidative stress.
- Exposure to NP-3 induced no biomarkers (including genotoxicity markers), suggesting that the induction of micronuclei observed in the MN assay was unlikely due to a direct genotoxic mechanism.

ToxTracker + ROS Scavengers

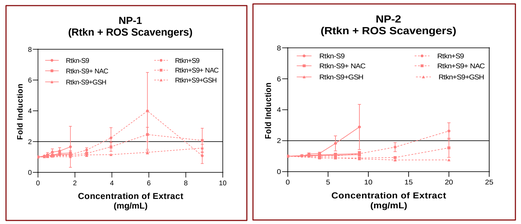


Figure 5: Activation of the DNA damage reporter Rtkn following exposure to increasing concentrations of NP-1 and NP-2 pouch extracts in presence of 10 mM ROS scavengers (NAC or GSH), in presence and absence of S9. The dark line in the graphs represents the two-fold induction level as the threshold for a positive ToxTracker classification.

- If indirectly genotoxic, activation of DNA damage response are expected to decrease in presence of ROS scavengers, GSH and NAC.
- Activation of Rtkn reporter was decreased below 2-fold with GSH, but not with NAC for NP-1, while for NP-2, activation of Rtkn reporter was reduced below 2-fold with both GSH and NAC. These observations are indicative of indirect genotoxicity by both NP-1 and NP-2 in the ToxTracker as well as the MN assay.

References: 1) OECD. (2020). OECD Guideline for Testing Chemicals Test Guideline 471, Bacterial Reverse Mutation Test; 2) OECD. (2016). OECD Guideline for Testing Chemicals Test Guideline 487, In vitro Mammalian Cell Micronucleus Test; 3) Brandsma, et. al (2020). *Toxicol. Sci.*177 (1); 202.

CONCLUSIONS

- ❖ **NP-3 was non-genotoxic in the ToxTracker Assay.**
- ❖ **Genotoxicity (Rtkn activation) was observed for NP-1 and NP-2 however, the response was preceded by high oxidative stress (Srxn1 and Blvrb).**
- ❖ **Addition of ROS scavengers (NAC and GSH) reduced genotoxicity (Rtkn activation), indicating both NP-1 and NP-2 were indirectly genotoxic due to oxidative stress and further suggesting that the observed positive response in the in vitro MN assay, was not due to direct genotoxic mechanisms.**
- ❖ **In vitro genotoxic responses to NP extracts may be driven by non-nicotine GRAS ingredients (e.g., cinnamaldehyde).**
- ❖ **New approach methodologies such as ToxTracker, can provide mechanistic insights into positive findings from standard in vitro genotoxicity assays, which may not always stem from direct genotoxic mechanisms.**
- ❖ **These insights supplement and help contextualize outcomes of traditional genotoxicity assays, strengthen overall weight of evidence and inform product stewardship decisions.**

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