# In vitro assessment of Tobacco-Free Nicotine Pouches reveals marked reductions in Toxicity when compared to Cigarettes.

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## 1. INTRODUCTION

Tobacco-free nicotine pouches (TFNPs) are an emerging category of nicotine-containing oral products. These products do not contain tobacco leaves, do not undergo combustion, and nicotine delivery is through the oral mucosa. As TFNPs don't contain or burn tobacco, research demonstrates that they contain fewer and substantially lower levels of the harmful chemicals found in cigarette smoke[1] and studies have shown that the reduced level of toxicants translates to reduced *in vitro* biological activity compared to cigarette smoke extracts[2,3,4,5]. In this study we tested a variety of TFNPs using three *in vitro* toxicological assays to demonstrate their reduced risk potential when compared to a cigarette smoke extract.

## 2. METHODOLOGY

### Test Products

- Twenty-one tobacco-free nicotine pouches (TFNP)
- 1R6F reference cigarette (University of Kentucky)


**Figure 1:** TFNPs

The nicotine pouches included a range of flavours and nicotine strengths.

**Table 1:** Summary of test products used in the study

| Test Article | Nicotine strength (mg nicotine/pouch) | Flavour direction |
|---|---|---|
| TFNP #1 | 4.08 | Menthol |
| TFNP #2 | 6.03 | Menthol |
| TFNP #3 | 7.22 | Mint/Lime |
| TFNP #4 | 9.63 | Red Berry/Vanilla |
| TFNP #5 | 9.63 | Watermelon |
| TFNP #6 | 9.63 | Orange |
| TFNP #7 | 9.63 | Peach |
| TFNP #8 | 9.63 | Liquorice |
| TFNP #9 | 10 | Citrus |
| TFNP #10 | 11.89 | Mint |
| TFNP #11 | 12 | Citrus/Mint |
| TFNP #12 | 12.03 | Menthol |
| TFNP #13 | 14 | Berry/Vanilla |
| TFNP #14 | 14.45 | Menthol |
| TFNP #15 | 14.54 | Menthol |
| TFNP #16 | 15.05 | Berry |
| TFNP #17 | 16 | Menthol |
| TFNP #18 | 16 | Sriracha/Lime |
| TFNP #19 | 17.65 | Red Berry/Vanilla |
| TFNP #20 | 18 | Menthol |
| TFNP #21 | 20 | Menthol |
| 1R6F | N/A | N/A |

### Generation of PBS Extracts

The extraction of OND was based on ISO 10993-12:2021, chapter 10. The closed TFNP were covered with 20 ml phosphate buffered saline (PBS) as an extraction medium to obtain an extraction ratio of 300mg/ml and agitated at 600rpm and room temperature for 1 hour. After centrifugation and filtration through 0.2µm sterile filters, aliquots of 550µl per extract were frozen at -80°C. Four independent extracts for each product were generated and nicotine content was determined by GC/FID.

### Biological Assessment

All of the in vitro techniques were performed according to the methodology outlined by Yu et al. [1]
All statistical analyses were performed using GraphPad Prism version 8.4.3.

**NRU**

The NRU assay was carried out in BEAS-2B and HepG2 cells; alongside negative and positive controls, TFNP extracts were added to cultures at concentrations in the range of 0.5-21mg PBS/ ml medium. 1R6F TPM was applied in the range of 0.005-0.05mg DMSO/ ml medium. Outcomes were compared on a concentration required to induce 20% ($EC_{20}$) and 50% cytotoxicity ($EC_{50}$) basis. Comparison was made to 1R6F for the TFNP using an ANOVA and multiple Dunnett's tests Pair-wise comparisons with Tukey's tests were carried out between nicotine pouch products.

**Ames**

Performed according to OECD Guideline 471 - Five *Salmonella typhimurium* strains were assessed in the bacterial reverse mutation (Ames) test, TA98, TA100, TA102, TA1535 and TA1537 (+/-S9). Alongside negative and positive controls, TFNP extracts were added to cultures at concentrations in the range of 1-5mg PBS/ plate; 1R6F TPM was obtained under ISO conditions and applied in the range of 0.025-0.125mg DMSO/ plate. Mutagenic activity was analysed using the slope of the dose-response (fold increase in revertants) using a non-threshold model and Dunnett's test.

**In Vitro Micronucleus (IVM)**

Performed according to OECD Guideline 487 - Three treatment schedules were applied in the micronucleus assay to Chinese hamster lung fibroblast V79 cells: short-term +S9, short term -S9 and long-term -S9. For the TFNPs extracts, cells were exposed to a range of concentrations between 2-5mg PBS/ ml medium, and for 1R6F, this range was 0.03-0.14mg DMSO/ ml medium dependent on treatment schedule (tested alongside negative and positive controls). Outcomes were assessed for significance using a Chi-Square analysis with Cochran-Armitage trend test.

## 3. RESULTS

### 3.1 NRU

- 12 out of the 21 TFNP extracts did not induce $EC_{50}$ values in the BEAS-2B cell line while 8 out of the 21 TFNP extracts did not induce $EC_{50}$ values in the HepG2 cell line (Extrapolated $EC_{50}$ values have been included in Figure 2).
- $EC_{20}$ values obtained for the TFNP extracts ranged between 32 and 618 times less cytotoxic than the 1R6F cigarette TPM when calculated on a nicotine basis.
- Variation in the $EC_{20}$ values was observed between TFNP products, but no correlation was observed with nicotine content or flavour (Figure 2). This variance was more pronounced for BEAS-2B.
- $EC_{50}$ values obtained from the TFNP extracts ranged between 50 and 160 times less cytotoxic than the 1R6F cigarette TPM when calculated on a nicotine basis.

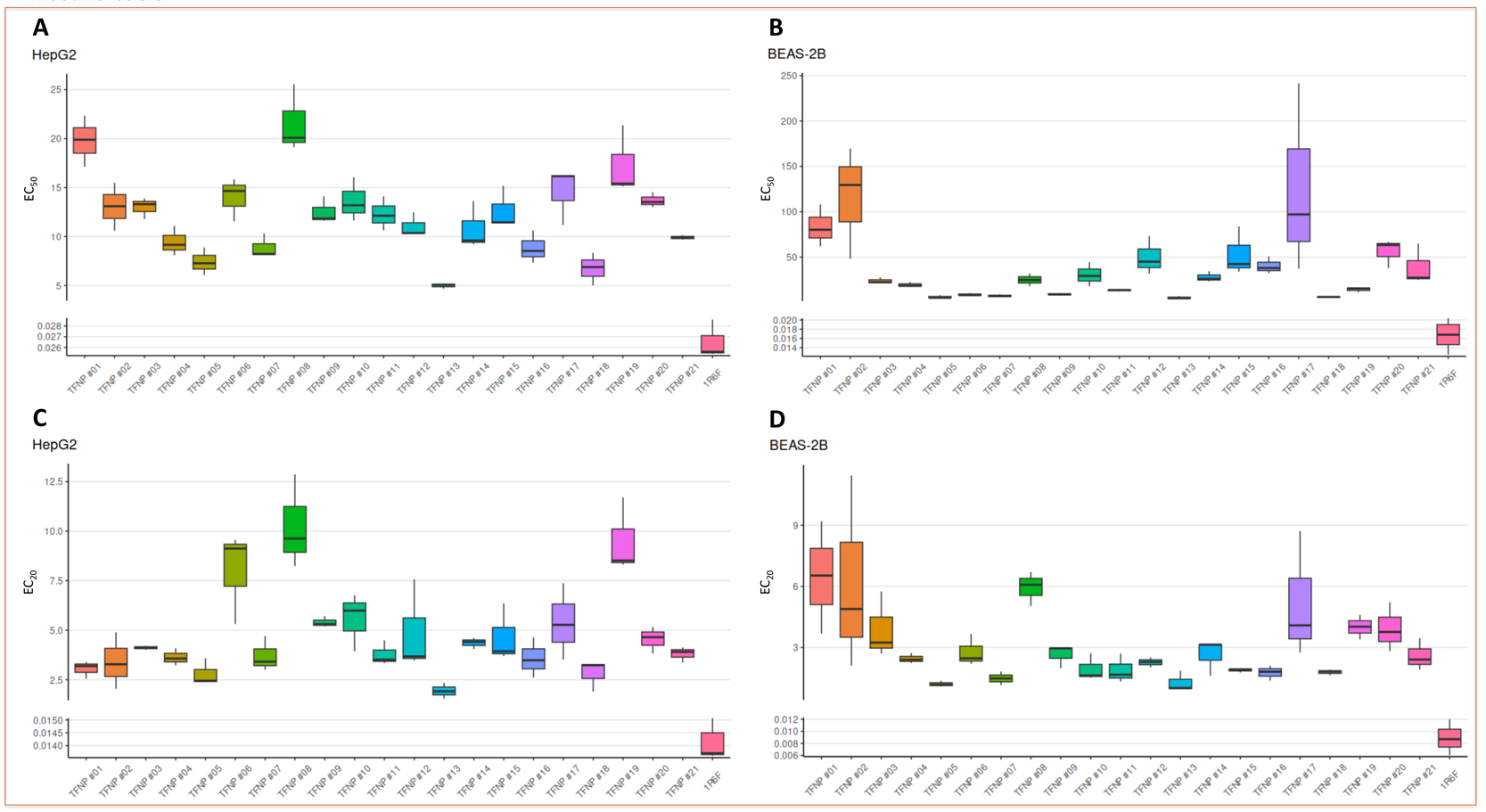


**Figure 2 (A-D):** Concentrations (mg extract/ml medium) required to induce 50% ($EC_{50}$) and 20% ($EC_{20}$) cytotoxicity in HepG2 and Beas-2B cells compared to negative control for the test article extracts.

### 3.2 Ames

- None of the TFNP extracts demonstrated any evidence of causing reproducible, dose-dependent, or statistically significant increases in the number of revertants with or without S9 (metabolic activation) mix in any of the test strains, therefore TFNP extracts did not meet the criteria to be classified as mutagenic under the test conditions.
- 1R6F TPM caused reproducible, dose-dependent statistically significant increases in the number of revertants in TA98 (+/-S9), TA100 (+/-S9) and TA1537 (+S9).
- Therefore, 1R6F TPM met the criteria to be classified as mutagenic under the test conditions.
- The difference in response between TFNP extracts and 1R6F TPM in Ames Assay can be observed in Figure 3.

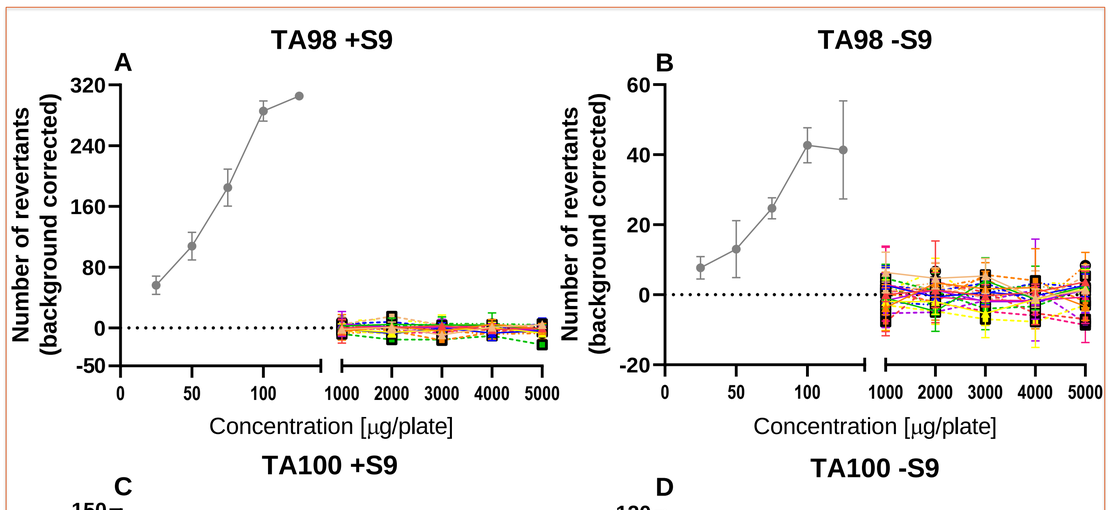


**Figure 3 (A-D):** Dose-specific mutagenicity in *Salmonella typhimurium* in TA98 and TA100 cell lines in the presence and absence of metabolic activation (S9) after exposure.

### 3.3 IVM

- None of the TFNP extracts induced dose-dependent, reproducible, or statistically significant increases in micronucleus frequencies as compared to the negative controls in any of the three treatments schedules applied and so did not meet the criteria to be classified as genotoxic under the test conditions (maximum concentrations: 5000 µg/ml in all treatment schedules).
- 1R6F TPM induced significant, dose-dependent and reproducible increases in micronucleus frequencies, therefore meeting the criteria to be classified as genotoxic under the test conditions (maximum concentrations: 120 µg/ml in ST -S9; 140 µg/ml in ST +S9; 90 µg/ml in LT -S9 ).

## 4. CONCLUSIONS

- In the NRU assay, 1R6F TPM induced higher cytotoxicity compared to TFNP extracts in HepG2 and BEAS-2B cell lines by 32 to 618-fold. There was no observed correlation between cytotoxicity and nicotine content of TFNP in the NRU assay for both cell lines tested.
- In the IVM and Ames assays the TFNP extracts were negative for genotoxicity and mutagenicity under the conditions of the test while in contrast 1R6F was found to meet the criteria to be classified as mutagenic and genotoxic.
- These results demonstrate that the reduced toxicant levels present in TFNP compared to cigarette smoke results in marked reductions of in vitro activity. This suggests that TFNPs have the potential to offer a harm-reduced alternative to smoking cigarettes and the potential to make a meaningful contribution to tobacco harm reduction.

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