Oral nicotine pouch extracts elicit substantially reduced *in vitro* toxicity compared to cigarette smoke extract

United Kingdom Environmental Mutagen Society conference, Dublin
2nd-5th July 2023

Visit our Scientific Research website
www.imperialbrandscience.com

Fiona Chapman[1]*, Sarah Jean Pour[2], Thomas Verron[1], Edgar Trelles Sticken[2], Roman Wieczorek[2], Ruby Morrissey[1], Matthew Stevenson[1]
[1]Imperial Brands PLC, 121 Winterstoke Road, Bristol, BS3 2LL, UK; [2]Reemtsma Cigarettenfabriken GmbH, An Imperial Brands PLC company, Albert-Einstein-Ring-7, D-22761, Hamburg, Germany
*Corresponding author's e-mail: Fiona.Chapman@uk.imptob.com

1. INTRODUCTION

Oral nicotine delivery (OND) is posited to offer a potentially reduced harm alternative for adult smokers who do not wish to stop consuming nicotine [1]; this is due to the absence, or substantially reduced levels, of toxicants associated with tobacco combustion. Within the OND category are traditional products, such as Scandinavian tobacco snus, and the newer innovation, tobacco-free nicotine pouches (TFNPs). Tobacco naturally contains some toxicants; TFNPs are believed to offer further tobacco harm reduction potential compared to snus as they do not contain tobacco leaf [2, 3]. Within the TFNP category, different flavours and nicotine strengths play an important role in providing adult smokers with optionality to support switching from cigarettes.

Figure 1: Tobacco-free nicotine pouch (not to scale)

2. AIM

Due to the relative novelty of the TFNP category, this study aimed to compare the *in vitro* toxicological outcomes for a range of TFNP flavours and nicotine strengths to a traditional tobacco snus product and the 1R6F reference cigarette. An established battery of regulatory assays (neutral red uptake (NRU), *in vitro* micronucleus and Ames tests) was employed for this assessment.

3. METHODS

Test articles

Twelve tobacco-free nicotine pouches (TFNPs) (from the ZoneX and Skruf Superwhite brands), one traditional Scandinavian tobacco snus product (Skruf brand) (all obtained from the manufacturer, Imperial Brands PLC) and the 1R6F reference cigarette (University of Kentucky) were used in this study. The nicotine pouches were a range of flavour directions and nicotine strengths.

Table 1: Summary of products used in the study. TFNP = Tobacco-free nicotine pouch

Product	Product nicotine concentration (mg nicotine/pouch)	Flavour direction
TFNP #1	5.8	Menthol
TFNP #2	7.22	Mint/ lime
TFNP #3	9.63	Berries
TFNP #4	10.1	Menthol
TFNP #5	11.26	Spearmint
TFNP #6	11.78	Mint
TFNP #7	13.22	Menthol
TFNP #8	13.39	Cassis/ mint
TFNP #9	14.45	Menthol
TFNP #10	16	Menthol
TFNP #11	18	Menthol
TFNP #12	20	Menthol
Snus	10.9	Menthol
1R6F Reference Cigarette	-	-

Extract generation

Extracts for application in the respective *in vitro* tests were generated from the pouch products by shaking 6g of product in 20ml of phosphate buffered saline (PBS) solution at 600rpm and 37°C for 1 hour; the extract was then filtered through 0.2µm sterile filters (ISO 10993-12:2021 [4]). Total particulate matter (TPM) from the 1R6F reference cigarette (conditioned according to ISO 3402 [5]) was generated by smoking using a Borgwaldt RM-20 D smoking machine to the ISO 20779:2018 smoking regime (55 mL puff volume/ 2 second puff duration/ 30s puff interval) [6]. Smoke was passed through a 92mm Cambridge filterpad to trap the TPM, which was extracted by shaking in dimethylsulphoxide (DMSO) for 20min at room temperature. The DMSO extract was then centrifuged through 0.45µm sterile filters. Nicotine was quantified within the extracts according to the methodology described by Yu *et al*. [1].

Neutral red uptake (NRU) cytotoxicity assay

The NRU assay was carried out in Beas-2B and HepG2 cells; alongside negative and positive controls, TFNP and snus extracts were added to cultures at concentrations in the range of 0.5-10mg PBS/ ml medium; 1R6F TPM was applied in the range of 0.005-0.05mg DMSO/ ml medium. The assay was carried out according to the methodology outlined by Yu *et al*. [1]. 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 nicotine pouch products using multiple Dunnett's tests. Pair-wise comparisons with Tukey's tests were carried out between nicotine pouch products to elucidate any trends in the outcomes.

Micronucleus assay

Three treatment schedules were applied in the micronucleus assay to Chinese hamster lung fibroblast V79 cells: short-term, +/-S9/ long-term -S9, and according to the methodology outlined by Yu *et al*. [1]. For the TFNPs and snus 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.

Ames test

Five *Salmonella* Typhimurium strains were assessed in the bacterial reverse mutation (Ames) test, TA98, TA100, TA102, TA1535 and TA1537 (+/-S9), according to the methodology outlined by Yu *et al*. [1]. Alongside negative and positive controls, TFNP and snus extracts were added to cultures at concentrations in the range of 1-5mg PBS/ plate; 1R6F TPM was 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 nonthreshold model and Dunnett's test.

4.RESULTS

4.1 Extract nicotine content

Table 2: Nicotine concentrations in the extracts used for application in the respective *in vitro* assays. TFNP = tobacco-free nicotine pouch

Product	NRU - Nicotine concentration range tested (µg/ml)	Micronucleus - Nicotine concentration range tested (µg/ml)	Ames - Nicotine concentration range tested (µg/plate)	Product	NRU - Nicotine concentration range tested (µg/ml)	Micronucleus - Nicotine concentration range tested (µg/ml)	Ames - Nicotine concentration range tested (µg/plate)
TFNP #1	8.5-170	11.4-28.4	17-85	***TFNP #8***	62.8-314	35.1-87.7	62.8-314
TFNP #2	11.95-239	16.4-40.8	7.9-39.3	***TFNP #9***	20.9-418	28.9-72.2	41.1-205.5
TFNP #3	5.6-112.7	23.2-58.0	10.9-54.5	***TFNP #10***	12.85-257	19.7-49.2	25-125
TFNP #4	12.55-251	16.8-41.9	25.1-125.5	***TFNP #11***	13.15-263	17.7-44.2	26.1-130.5
TFNP #5	52.8-264	40.6-101.5	52.8-264	***TFNP #12***	18-360	25.1-62.8	35.3-176.5
TFNP #6	55.2-276	35.5-88.7	55.2-276	***Snus***	12.95-259	17.3-43.1	25.9-129.5
TFNP #7	11.4-227	37.4-93.5	20.7-103.3	***1R6F Reference Cigarette***	0.0675-0.675	0.03-7.2	0.34-1.69

- Extraction yielded a range of nicotine concentrations, dependent on product, however on a nicotine basis, concentrations applied for 1R6F were generally lower than those for the TFNP and snus products (**Table 2**)

4.2 Cytotoxicity (NRU) outcomes

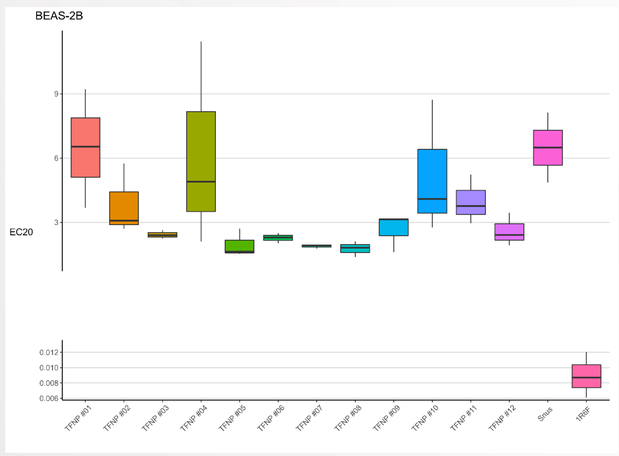

Figure 2: Concentrations (mg extract/ml medium) required to induce 20% cytotoxicity in Beas-2B cells compared to negative control (EC_{20}) for the test article extracts.

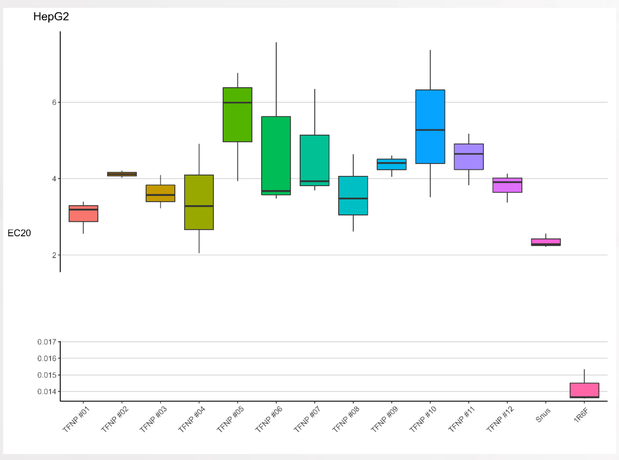

Figure 3: Concentrations (mg extract/ ml medium) required to induce 20% cytotoxicity in HepG2 cells compared to negative control (EC_{20}) for the test article extracts.

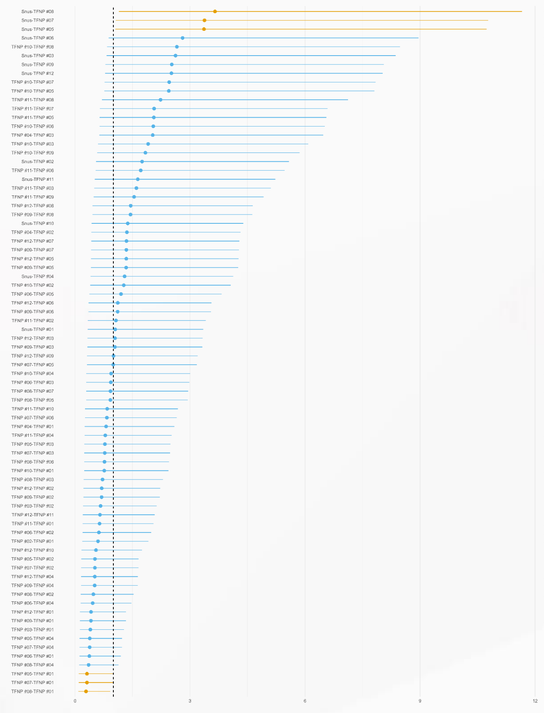

Figure 4: Pair-wise multiple comparisons of EC_{20} values induced by the TFNP and snus extracts in Beas-2B cells, carried out using Tukey's tests. Data are expressed as a ratio of mean EC_{20} levels. Orange bars indicate significant deviations

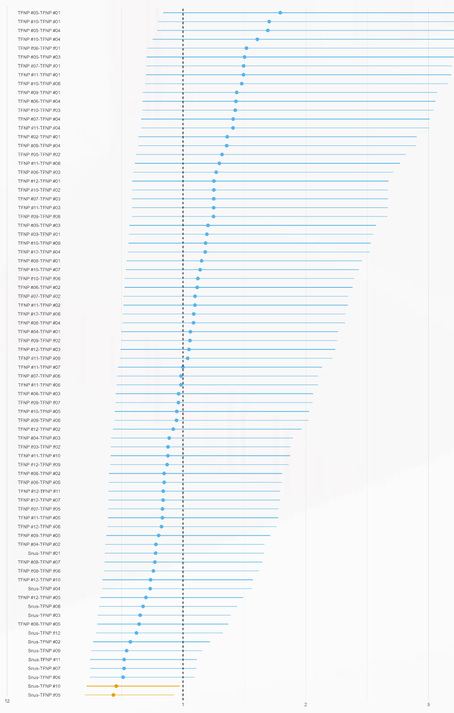

Figure 5: Pair-wise multiple comparisons of EC_{20} values induced by the TFNP and snus extracts in HepG2 cells, carried out using Tukey's tests. Data are expressed as a ratio of mean EC_{20} levels. Orange bars indicate significant deviations

- Products were compared on an EC_{20} basis as the majority of nicotine pouch products (TFNPs/ snus) did not achieve an EC_{50} with the concentrations tested
- Overall, when compared to 1R6F on a nicotine basis, the TFNP and snus extracts demonstrated substantially reduced cytotoxicity (for all comparisons to 1R6F using a Dunnett's test, $p<0.001$) (**Figures 2** and **3**) – up to a 99.9% reduction for Beas-2B and 99.8% for HepG2
- Although some variation was observed between TFNP products, this did not appear to correlate with flavour direction nor nicotine content, and no trends were observed between the two cell lines
- Nicotine pouch comparisons in Beas-2B produced slightly more significant differences between products than in HepG2 (**Figures 4** and **5**)

4.3 Micronucleus assay outcomes

- Under the test conditions, none of the TFNP extracts nor the snus extract induced significant, dose-dependent reproducible increases in micronucleus frequencies
- In contrast, 1R6F TPM induced significant, dose-dependent reproducible increases in micronucleus frequencies in the V79 cells under the three treatment schedules applied

4.4 Ames test outcomes

- Under the test conditions, the TFNP and snus extracts did not cause any significant, dose-dependent, reproducible increases in revertants. This was observed across all five *S.* Typhimurium strains (+/-S9)
- 1R6F TPM induced positive responses in TA98 and TA100 (+/-S9) and TA1537 (+S9), however was negative in TA102 and TA1535 (+/-S9) and TA1537 (-S9)

5. CONCLUSIONS

- The extracts applied ranged in nicotine concentration, however 1R6F elicited effects at far lower nicotine concentrations, suggesting the presence of combustion-related toxicants contributed to toxicological outcomes
- When applied to two cell lines in the NRU assay, the TFNP and snus products demonstrated substantially reduced cytotoxicity compared to 1R6F
- In the NRU assay, there were no clear trends based on pouch nicotine content nor flavour direction. Outcomes were consistent across the two cell lines tested, suggesting a potential role of metabolism of the extracts by HepG2 on cytotoxicity profile. Further mapping of entire product composition against cytotoxicity outcomes in the two cell lines may increase understanding of the drivers of effect, however, overall, differences between nicotine pouch products (for respective cell lines) were largely insignificant
- In the micronucleus and Ames assays, the TFNP and snus extracts demonstrated consistently negative results under the test conditions; in contrast, 1R6F was genotoxic in V79 (+/-S9) and mutagenic in the TA98, TA100 (+/-S9) and TA1537 (+S9) strains
- Overall, both the TFNPs (irrespective of nicotine strength or flavour direction) and the snus product consistently demonstrated substantial reductions in *in vitro* toxicity compared to the 1R6F reference cigarette, supporting the tobacco harm reduction potential of these discrete nicotine pouch categories

1. Yu *et al*. (2022). Preclinical Assessment of Tobacco-Free Nicotine Pouches Demonstrates Reduced *In Vitro* Toxicity Compared with Tobacco Snus and Combustible Cigarette Smoke. Applied In Vitro Toxicology, 8(1), 24–35, DOI: 10.1089/aivt.2021.0020
2. Azzopardi *et al*. (2022). Chemical characterization of tobacco-free "modern" oral nicotine pouches and their position on the toxicant and risk continuums, Drug and Chemical Toxicology, 45(5), 2246-2254, DOI: 10.1080/01480545.2021.1925691
3. Miller-Holt *et al*. (2022). *In vitro* evaluation of mutagenic, cytotoxic, genotoxic and oral irritation potential of nicotine pouch products. Toxicology Reports, 9, 1316-1324, DOI: 10.1016/j.toxrep.2022.06.008
4. International Organization for Standardization. ISO 10993-12:2021. Biological Evaluation of Medical Devices—Part 12: Sample Preparation and Reference Materials. Geneva, Switzerland: International Organization for Standardization; 2021
5. International Organization for Standardization. ISO 3402:2023. Tobacco and tobacco products—Atmosphere for conditioning and testing. https://www.iso.org/standard/28324.html
6. International Organization for Standardization. ISO 20779:2018. Cigarettes—Generation and Collection of Total Particulate Matter Using a Routine Analytical Smoking Machine with an Intense Smoking Regime. Geneva, Switzerland: International Organization for Standardization; 2018