P02-06

Assessing Innovation in Nicotine Pouches: In Vitro Evidence Supporting Reduced Toxicological Activity of New Pouch substrate blend

Eurotox 2026, Vienna 13-16 September 2026

Thomas Withers[1], Roman Wieczorek[2], Edgar Trelles Sticken[2], Sarah Jean Pour[2], Konstantinos Papikinos[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

FIND OUT MORE

INTRODUCTION

Smoking is a serious cause of disease in smokers including lung cancer, heart disease and emphysema. The greatest risk of smoking related diseases comes from burning tobacco and inhaling smoke containing around 7,000 chemicals[1]. For adults who smoke who would otherwise continue to smoke, nicotine pouches (NP) that do not burn tobacco have the potential to reduce exposure to harmful chemicals[2]. Unlike traditional tobacco products, NP do not burn tobacco and do not contain tobacco leaf. In addition, they deliver nicotine orally via the gum membranes, meaning lung-related toxicity and disease risks are not to be expected. As manufacturers introduce new pouch blends, it is important to evaluate whether innovations maintain the reduced in vitro toxicological profile observed in previous formulations. In this study all products were evaluated for cytotoxicity, genotoxicity and Mutagenicity.

Methodology

Test products

- Twenty-five NP produced by Imperial Brands PLC for the EU Markets
- 1R6F reference cigarette (University of Kentucky)

Table 1. Summary of test products.

Test Article	Nicotine strength (mg nicotine/pouch)	Flavour direction	Base blend
NP #1	4.08	Menthol	Previous Blend
NP #2	6.03	Menthol	Previous Blend
NP #3	7.22	Mint/Lime	Previous Blend
NP #4	9.63	Red Berry/Vanilla	Previous Blend
NP #5	9.63	Watermelon	Previous Blend
NP #6	9.63	Orange	Previous Blend
NP #7	9.63	Peach	Previous Blend
NP #8	9.63	Liquorice	Previous Blend
NP #9	10.00	Citrus	Previous Blend
NP #10	11.89	Mint	Previous Blend
NP #11	12.00	Citrus/Mint	Previous Blend
NP #12	12.03	Menthol	Previous Blend
NP #13	14.00	Berry/Vanilla	Previous Blend
NP #14	14.45	Menthol	Previous Blend
NP #15	14.54	Menthol	Previous Blend
NP #16	15.05	Berry	Previous Blend
NP #17	16.00	Menthol	Previous Blend
NP #18	16.00	Sriracha/Lime	Previous Blend
NP #19	17.65	Red Berry/Vanilla	Previous Blend
NP #20	18.00	Menthol	Previous Blend
NP #21	20.00	Menthol	New Blend
NP #22	10.70	Berry	New Blend
NP #23	10.70	Mint	New Blend
NP #24	16.08	Orange	New Blend
NP #25	16.08	Berry	New Blend
1R6F	N/A	N/A	N/A

NP extracts

NP extracts were prepared using extraction principles adapted from ISO 10993-12:2021, Section 10. Four independent extracts were prepared for each product at an extraction of 300mg/ml, with nicotine concentrations subsequently quantified using gas chromatography with flame ionization detection (GC-FID).

NRU

The NRU assay was conducted in BEAS-2B and HepG2 cells to assess cytotoxicity. Test samples included NP extracts at concentrations ranging from 0.5–21 mg PBS/mL culture medium and 1R6F TPM at concentrations ranging from 0.005–0.05 mg DMSO/mL culture medium. Negative and positive controls were included in each test. Cytotoxic potency was compared using the concentrations required to induce 20% and 50% cytotoxicity (EC_{20} and EC_{50}, respectively). Statistical comparisons between NP products and 1R6F were performed using one-way ANOVA followed by Dunnett's multiple comparison test. Pairwise comparisons between NP products were conducted using Tukey's test.

IVM

The IVM assay was conducted in accordance with OECD Guideline 487[3] using Chinese hamster lung fibroblast (V79) cells for NP 1-21 and TK6 cell lines for NP 22-25. Three treatment conditions were evaluated: short-term exposure with metabolic activation with and without S9 and long-term exposure without metabolic activation (−S9). NP extracts were tested at concentrations ranging from 2–5 mg NP extracts/mL culture medium, while 1R6F TPM was tested at concentrations ranging from 0.03–0.14 mg DMSO/mL culture medium, depending on the treatment schedule. Negative and positive controls were included in each test. Micronucleus frequency data were analysed using Chi-square tests, with dose-related trends assessed using the Cochran–Armitage trend test.

Ames

The Ames assay was performed in accordance with OECD Guideline 471[4]. using five Salmonella typhimurium strains (TA98, TA100, TA102, TA1535 and TA1537), both in the presence and absence of metabolic activation (±S9). NP extracts were tested at concentrations ranging from 1–5 mg PBS/plate, while 1R6F TPM, generated under ISO smoking conditions, was tested at concentrations ranging from 0.025–0.125 mg DMSO/plate. Negative and positive controls were included in each test. Mutagenic activity was evaluated using a non-threshold dose–response model, with the slope of the revertant response (fold increase in revertants) used as the primary endpoint. Statistical significance was assessed using Dunnett's test.

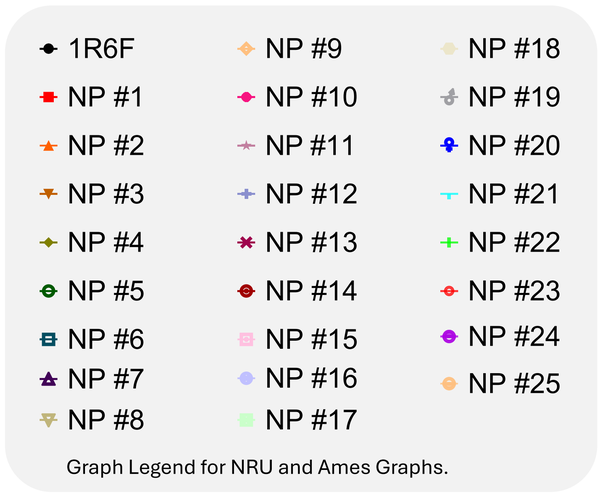
Results

NRU

A total of 12 out of the 25 NP extracts did not induce EC50 values in the BEAS-2B cell line. Additionally,, 10 out of the 25 NP extracts did not induce EC50 values in the HepG2 cell line.

EC20 values obtained for the NP extracts ranged between 31 and 618 times less cytotoxic than the 1R6F on a nicotine basis.

Variation in the EC_{20} and EC_{50} values was observed between NP products, but no correlation was observed with nicotine content, base blend or flavour (Figure 1).



Graph Legend for NRU and Ames Graphs.

IVM

None of the NP extracts for both new and old blend induced a dose-dependent, reproducible or statistically significant increase in micronucleus frequencies as compared to the negative controls in any of the three treatments schedules applied. They did not therefore meet the criteria to be classified as genotoxic under the test conditions (maximum concentrations:5000 µg/ml in all treatment schedules).

AMES

Both new and old blend NP extracts demonstrated no evidence of causing reproducible, dose-dependent, and statistically significant increases in the number of revertants (+/-S9) across the test strains. Therefore, NP 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).

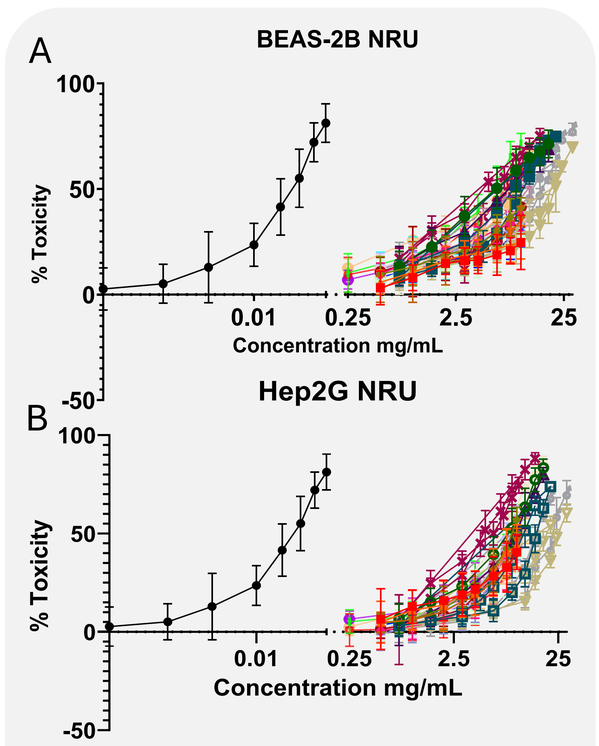


Figure 1(A,B).Cytotoxicity of NP extracts in HepG2 and BEAS-2B cells compared with 1R6F cigarette extract (black), expressed as percentage cytotoxicity at the indicated concentrations (mg extract/mL culture medium).

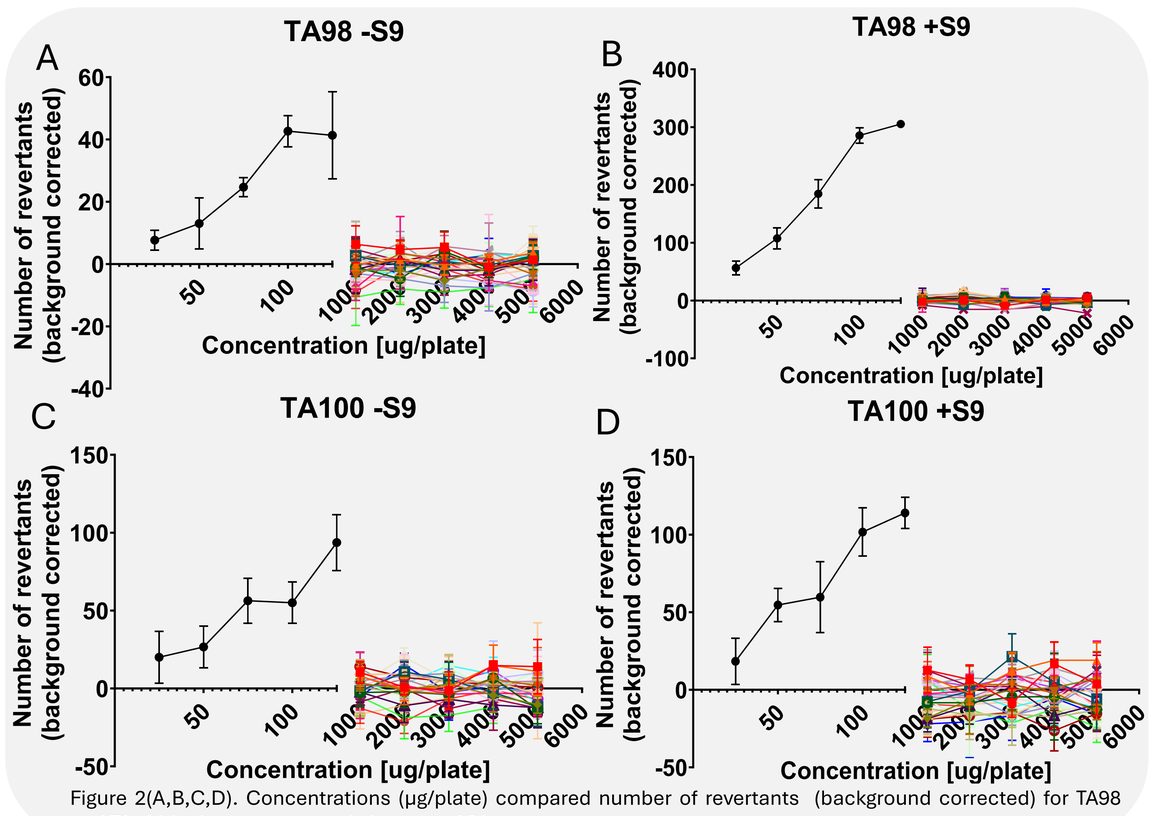


Figure 2(A,B,C,D). Concentrations (µg/plate) compared number of revertants (background corrected) for TA98 and TA100 in the presence and absence of S9.

CONCLUSIONS

- Across the three assays, 1R6F met the criteria for classification as cytotoxic, genotoxic and mutagenic.
- IVM and Ames assays showed that NP extracts were negative for genotoxicity and mutagenicity under the conditions of the test.
- NRU assay showed NP extracts and 1R6F TPM both caused cytotoxicity, with 1R6F TPM showing 31- to 618-fold greater potency in HepG2 and BEAS-2B cells.
- There was no observed correlation across the three assays that nicotine content, flavour, or base blend among NPs lead to any correlation in genotoxic, mutagenic and cytotoxic potential.
- This results show new blend NPs continue to offer a harm-reduced alternative to smoking cigarettes and the potential to make a meaningful contribution to tobacco harm reduction.

REFERENCES

1. World Health Organization. (2025). Tobacco. In *World Health Organization*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/tobacco. Accessed 11 August 2026
2. 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.
3. OECD Guideline for the Testing of Chemicals. Draft proposal for a new guideline 487: In vitro Micronucleus Test.
4. OECD Guideline for the Testing of Chemicals. Test number 471: Bacterial Reverse

IMPERIAL BRANDS SCIENCE

zone