# Oral-Immune Co-Culture Model Reveals Significantly Lower Toxicity of Oral Nicotine Pouches Compared to Cigarettes

FIND OUT MORE

SOT 65th Annual Meeting & ToxExpo March 22-25th 2026, Abstract number 3461, H612, ToxExpo, Hall B

M. Stevenson[1*], L. Scott[2], J. Kelsall[2], E. Hoffman[2], V. Hutter[2], F. Chapman[1], R. Wieczorek[3], E. Trelles-Sticken[3], S. Pour[3], T. Evenburg[3], and L. Simms[1]. [1]Imperial Brands PLC, Bristol, UK; [2]ImmuONE Ltd, Stevenage, United Kingdom; and [3]Reemtsma Cigarettenfabriken GmbH, An Imperial Brands PLC company, Hamburg, Germany.*Corresponding author's e-mail: Matthew.Stevenson@impbrands.com

## INTRODUCTION

Oral Nicotine Pouches (ONP) are an emerging category of nicotine products gaining popularity with adult smokers. As ONP don't burn tobacco, research demonstrates they contain significantly fewer and substantially lower levels of harmful chemicals compared to cigarette smoke, therefore offering adult smokers a potentially less harmful way to consume nicotine. Initial in vitro investigations of these products reveal marked reductions in cytotoxicity when compared to cigarette smoke extracts (>200 fold less cytotoxic) and no genotoxicity (under the conditions of Ames and In Vitro Micronucleus tests), see Figure 1.

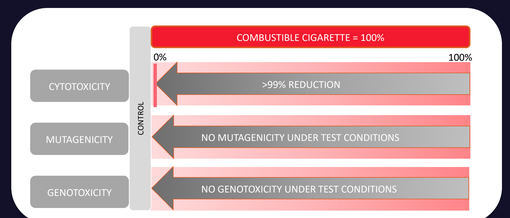



Figure 1: Reductions in biological activity of ONP vs cigarettes. Based on a Zone X #2 product (5.8 mg/pouch) compared to a cigarette TPM. See Yu *et al.*, 2022

Building on this data, we sought to better reflect the complex cellular interactions occurring at the oral mucosal interface, by employing a novel oral-immune co-culture model to assess the impact of ONP; tobacco-based Snus and cigarette smoke extracts.

### 2.1 Test Articles

- 1R6F Reference Cigarette (University of Kentucky)
- Snus, Tobacco based, Skruf No2 fresh S2 (10mg/pouch nicotine)
- ONP, Skruf Super White No52 Fresh S2 (5.8mg/pouch nicotine)

Both ONP and Snus were obtained from the EU market.

### 2.2 Pouch Extract Preparation

Snus and ONP products were extracted following the method outlined by Yu *et al.* (2022), utilising PBS for the oral-immune coculture test. Nicotine content was quantified, and extracts were stored at −70 ± 6 °C


Figure 2: Cross-sectional diagram of co-culture model

### 2.3 Total particulate matter (TPM) and Smoke bubbled PBS Extracts

1R6F reference cigarettes were smoked under Health Canada Intense conditions. TPM was collected on Cambridge filter pads and extracted with DMSO. For smoke bubbled extracts, smoke was bubbled through PBS (adapted from Czekala et al., 2020). Nicotine content was quantified, and extracts were stored at −70 ± 6 °C.

### 2.4 Cell Culture

With regards to the oral-immune coculture, ImmuPHAGE™ was seeded onto black µ-clear 24-well plates at a density of 2.5 x $10^5$ viable cells/mL (500 µL/well) and incubated at 37°C in an atmosphere of 5% CO2 (standard conditions) until required. On the day of dosing, the TR146 cells on Transwell® inserts (supplied by SkinEthic™) were combined with wells containing ImmuPHAGE™ cells. See Figure 2 for cross-sectional diagram. The biological assessment of the coculture was performed by ImmuONE Ltd.

### 2.5 PrestoBlue™ Cell Viability and barrier function assessment of TR146 cells

Cell viability was measured using PrestoBlue reagent, which detects metabolic activity via resazurin reduction. Transwell® inserts were incubated with 180 µL medium and 20 µL PrestoBlue at room temperature for 2 h. Fluorescence was read at 520 nm excitation and 580–640 nm emission using a Promega GloMax™ plate reader. Barrier function was assessed through trans-epithelial electrical resistance (TEER) which was recorded using EVOM2 chopstick electrodes. Results were normalised to the insert area and plotted relative to the appropriate vehicle control. TEER was measured before and after test item exposure. TEER values were below 50 Ω.cm² for all samples tested prior to dosing.

### 2.6 Immunostaining of Immune cells

ImmuPHAGE™ cells were stained with a dye cocktail containing Hoechst 33342 (nuclei), MitoTracker Red (active mitochondria), caspase 3/7 (early apoptosis) and imaged immediately, followed by fixing and staining with Cell Mask Deep Red (cytoplasm to identify vacuoles). Images were acquired using an InCell Analyser 6000 system (GE Healthcare). Each sample was imaged using 36 fields representing in total between 500 to 1500 cells per well. Images were analysed and quantified for cell morphology (cell area, number of vacuoles and total area of vacuolation) and cell health (mitochondrial and caspase 3/7 activity).

## 3.0 RESULTS:

## 3.1 EPITHELIAL CELL HEALTH

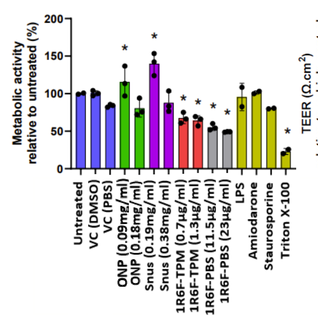



**Figure 3: PrestoBlue activity (cell viability) after 48h treatment with test items and controls.** Data are presented as a mean +/- SD of n=3 wells. Statistical significance was determined using one-way ANOVA with Dunnet's multiple comparison test and marked as *p<0.05.

**Summary:**
**ONP & Snus:** Metabolic activity increased significantly with low-dose treatments but lacked a dose-response. **1R6F-TPM/PBS:** both concentrations caused significant reductions in metabolic activity.

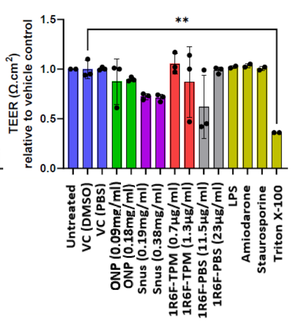



**Figure 4: Assessment of epithelial barrier function after exposure to test items and controls for 48 h.** Results were normalised to the insert area and plotted relative to the appropriate vehicle control.

**Summary:**
No significant reductions in TEER were recorded for any test articles. Positive control caused a significant reduction of TEER.

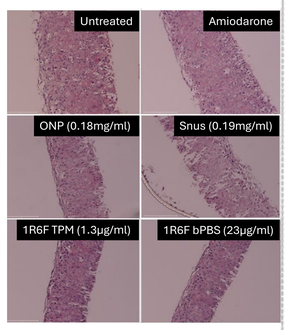
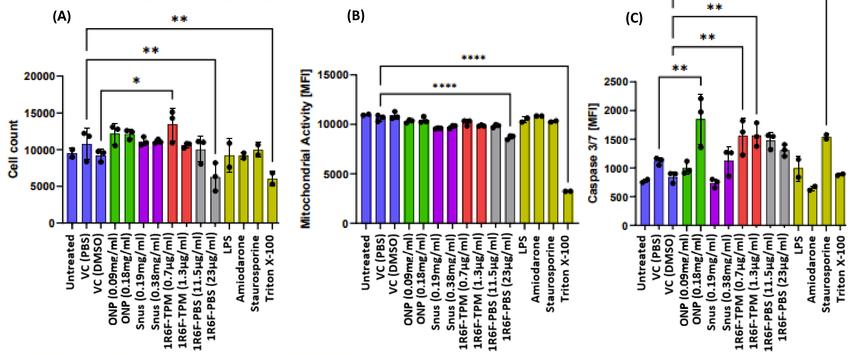



**Figure 5: Assessment of epithelial barrier function using histopathology exposure to test items and controls for 48 h.**

**Summary:**
All exposed tissues, demonstrated a thickness within normal range, suggesting a lack of marked cytotoxicity

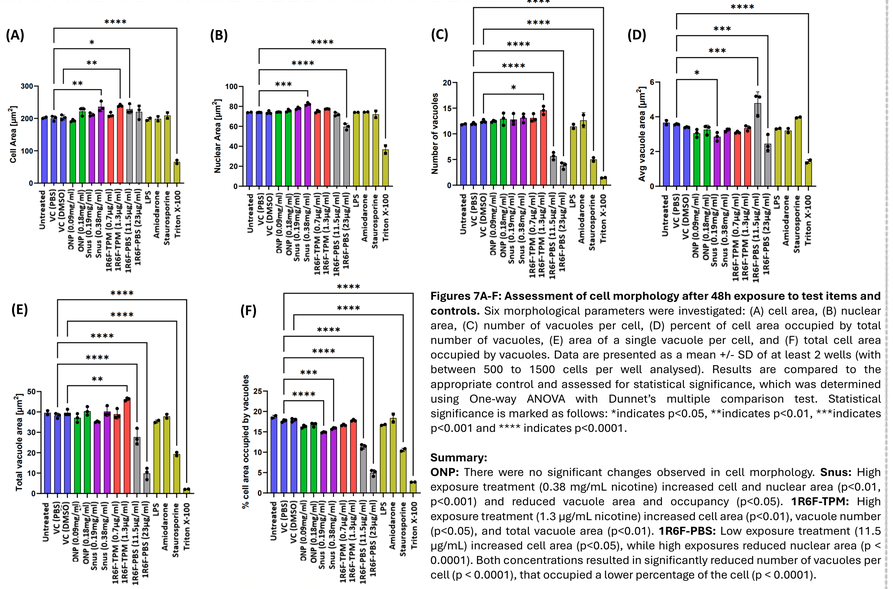
## 3.2 MACROPHAGE CELL HEALTH




**Figures 6A-C: Assessment of cell health after 48h exposure to test items and controls. Three cell health parameters were investigated: (A) cell count, (B) mitochondrial activity, and (C) early apoptosis.** Data are presented as a mean +/- SD of at least 2 wells (with between 500 to 1500 cells per well analysed). Results are compared to the appropriate control and assessed for statistical significance, which was determined using One-way ANOVA with Dunnet's multiple comparison test. Statistical significance is marked as follows: *indicates p<0.05, **indicates p<0.01, ***indicates p<0.001 and **** indicates p<0.0001.

**Summary:**
**ONP:** High exposure treatment (0.18 mg/mL nicotine) significantly increased caspase 3/7 activity (early apoptosis). **Snus:** No significant effects on cell health. **1R6F-TPM:** Caspase 3/7 activity (early apoptosis) increased at both concentrations (p<0.01). **1R6F-PBS:** High exposure treatment (23 µg/mL nicotine) reduced cell count (p<0.01) and mitochondrial activity (p<0.0001).

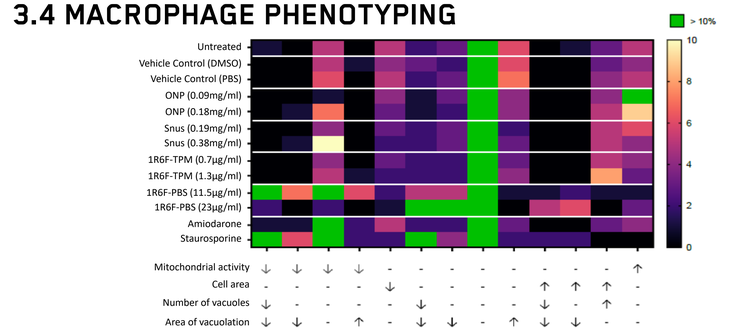
## 3.3 MACROPHAGE CELL MORPHOLOGY




**Figures 7A-F: Assessment of cell morphology after 48h exposure to test items and controls.** Six morphological parameters were investigated: (A) cell area, (B) nuclear area, (C) number of vacuoles per cell, (D) percent of cell area occupied by total number of vacuoles, (E) area of a single vacuole per cell, and (F) total cell area occupied by vacuoles. Data are presented as a mean +/- SD of at least 2 wells (with between 500 to 1500 cells per well analysed). Results are compared to the appropriate control and assessed for statistical significance, which was determined using One-way ANOVA with Dunnet's multiple comparison test. Statistical significance is marked as follows: *indicates p<0.05, **indicates p<0.01, ***indicates p<0.001 and **** indicates p<0.0001.

**Summary:**
**ONP:** There were no significant changes observed in cell morphology. **Snus:** High exposure treatment (0.38 mg/mL nicotine) increased cell and nuclear area (p<0.01, p<0.001) and reduced vacuole area and occupancy (p<0.05). **1R6F-TPM:** High exposure treatment (1.3 µg/mL nicotine) increased cell area (p<0.01), vacuole number (p<0.05), and total vacuole area (p<0.01). **1R6F-PBS:** Low exposure treatment (11.5 µg/mL) increased cell area (p<0.05), while high exposures reduced nuclear area (p < 0.0001). Both concentrations resulted in significantly reduced number of vacuoles per cell (p < 0.0001), that occupied a lower percentage of the cell (p < 0.0001).

## 3.4 MACROPHAGE PHENOTYPING




**Figures 8: Heatmap indicating phenotypic assessment of alveolar macrophages.** ImmuPHAGE™ was exposed to test items and controls for 48 h. Four cell characteristics (mitochondrial activity, cell area, vacuole number per cells and area of cell occupied by vacuoles) were expressed at three levels generating 81 possible phenotypes. Phenotypes above 5% were selected and presented as a heatmap. Each square represents the % of the cell population with that given phenotype in one experiment (at least 2 wells with between 500 to 1500 cells per well analysed). The colour gradient sets the lowest value for each given parameter in the heat map (black 0%), highest value (green above 10%) and mid-range values (yellow 10%) with a corresponding gradient between these extremes. Values in each block represent % of cell population displaying given phenotype.

**Summary:**
**ONP:** There was an increase in the percentage of cells displaying increased mitochondrial activity with moderate levels of morphological parameters at the lower concentration (0.09 mg/mL nicotine), but exposure to the higher concentration (0.18 mg/mL nicotine) resulted in a rise in the percentage of cells associated with lower mitochondrial activity, and phenotypes with increased cell area and increased number of vacuoles. **Snus:** resulted in a subpopulation with decreased or increased mitochondrial activity without morphological changes, and with a phenotype associated with increased cell area and vacuole numbers. These dose-dependent responses were similar to amiodarone induced phenotypes and suggested cells changing their morphology towards more foamy-like (lipid-laden) appearance. **1R6F-TPM:** Displayed a very similar dose-dependent profile, but with even more profound vacuolation (an increase in phenotype associated with bigger cell size and high number of vacuoles). **1R6F-PBS:** The phenotype resulting from this test article (especially at 11.5 µg/mL nicotine) was consistent with a pro-apoptotic mechanism of action, akin to staurosporine-induced apoptosis.

## CONCLUSION

- Both cigarette smoke extracts elicited dose-dependent toxicological effects. The 1R6F-TPM increased caspase 3/7 activity, displayed reduced PrestoBlue metabolism and induced a foamy macrophage phenotype (enlarged cells with increased numbers of vacuoles), whilst 1R6F-PBS caused cell death at the highest concentration, marked by reduced cell count, low PrestoBlue metabolic activity and smaller cells without vacuoles.
- For the ONP extract, the highest test concentration induced an increase in caspase 3/7 activation (apoptosis), with no significant changes in macrophage morphology at any concentration tested. The responses to ONP extracts were observed at concentrations approximately 7 to 250-fold higher than those elicited by Cigarette smoke extracts.
- The assessed Snus extracts induced dose-dependant changes similar to those seen with ONP but with no caspase activation, suggesting a different mechanism of toxicity than apoptosis. The cells were less active at the higher concentration with some foamy-like changes in phenotypes (increased cell area with more vacuoles). Note: Typical nicotine concentrations in the saliva of smokeless-tobacco users have been measured at approximately 73 µg/mL (Zanetti *et al.*, 2019).
- Overall, the results suggest that the smokeless products have the potential to offer a harm reduction alternative to smoking cigarettes and the potential to make a meaningful contribution to tobacco harm reduction. Further optimisation of the test system, including a wider range of test article concentrations and exposure durations is are required for future assessment of these products.

## REFERENCES

1. Yu, F., Rudd, K., Pour, S. J., Sticken, E. T., Dethloff, O., Wieczorek, R., *et al.* (2022). Preclinical assessment of nicotine pouches demonstrates reduced in vitro toxicity compared with tobacco snus and combustible cigarette smoke. Appl. Vitro Toxicol. 8, 24–35. doi:10.1089/aivt.2021.0020
2. Czekala L, Chapman F, Simms L, *et al.* The in vitro ToxTracker and Aneugen Clastogen Evaluation extension assay as a tool in the assessment of relative genotoxic potential of e-liquids and their aerosols. Mutagenesis. 2021;36(2):129-142. doi:10.1093/mutage/geaa033
3. Zanetti F, Sewer A, Titz B, *et al.* Assessment of a 72-hour repeated exposure to Swedish snus extract and total particulate matter from 3R4F cigarette smoke on gingival organotypic cultures. Food Chem Toxicol 2019:125;252–270

IMPERIAL BRANDS SCIENCE