Multi-endpoint *in vitro* toxicological assessment of snus and tobacco free nicotine pouch extracts

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Introduction

Tobacco free nicotine pouches (NPs) are a nicotine containing product similar in appearance and concept to Swedish snus. These products aim to further tobacco harm reduction by removing the exposure to tobacco related toxicants. NPs, being a fairly new product category, have limited *in vitro* data. A three-step approach was taken to analyse the biological effects of NPs and snus extracts *in vitro*.

Methodology

1 Study Design

1. ***In vitro* disease assessment**
2. **Regulatory toxicological assessment**
3. **Potential signalling pathways or inflammatory response**

Figure 1. A three-step approach to assess the biological effects of tobacco-free nicotine pouches and snus.

2 Sample Preparation

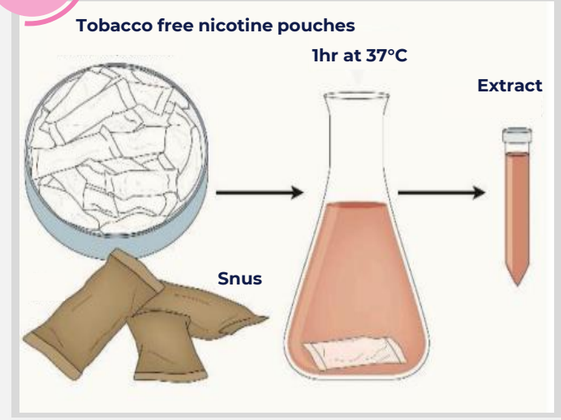


Figure 2. Sample preparation for tobacco-free nicotine pouches and snus in cell culture media.

3 Test Articles

	Velo*			Commercial Comparator	CORESTA Refence Product 1.1
Product Type	Tobacco-Free Nicotine Pouch				Snus
Source	British American Tobacco			Nordic Spirit	CORESTA
Flavour	Berry Frost	Tropic Breeze	Ice Cool	Wild berry & Bergamot	-
Nicotine Strength (per pouch)	4mg	4mg	10mg	6mg	8mg
PCode	LYFT_BF04	LYFT_TB04	LYFT_IC10	NDSP_BW06	CRP1.1

** VELO previously marketed as LYFT*

Table 1. The test products used and assessed

4 *In vitro* Assays

	ToxTracker	NRU	Ames	MLA	Signalling
Cell Line	Mouse embryonic stem cell (mESC)	Mouse fibroblasts (Balb/c 3T3 clone A31)	Salmonella typhimurium (TA98, TA100, TA1535, TA1537 and TA102)	Mouse lymphoma cells (L5178Y tk+/-)	NCI-H292 lung carcinoma cells
Readout	GFP Induction	Induction of cell death	Mutation Frequency	Induction of mutations	Cytokine release
Citation	Smart et al 2022 (1)	OECD Test Guideline No. 129 (2)	OECD Test Guideline No. 471 (3)	OECD Test Guideline NO. 490 (4)	Tsolakos et al in submission (5)

Table 2. Summary of In vitro assays used in this study

Results: Regulatory toxicological assessment

Regulatory Assay		Velo™			Commercial Comparator	CORESTA Refence Product 1.1
		LYFT_BF04	LYFT_TB04	LYFT_IC10	NDSP_BW06	CRP1.1
NRU (Cytotoxicity)		X	X	X	X	X
Ames (Mutagenicity)		X	X	X	X	X
MLA (Genotoxicity)	3 hrs –S9	X	X	X	X	X
	3 hrs +S9	X	X	X	X	X
	24 hrs –S9	?	X	X	X	✓

X denotes negative; ? denotes equivocal and ✓ denotes positive response

Table 3. Summary of NRU, Ames and MLA results

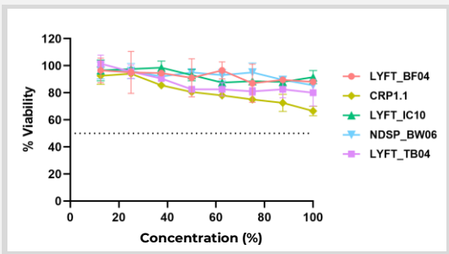


Figure 4. The percentage viability of Balb/c 3T3 fibroblast cells after exposure to the five test article extracts (%). Cytotoxicity was determined by neutral red uptake. Data are mean ± SD from two independent experiments. Grey dotted line indicates 50% cell viability.

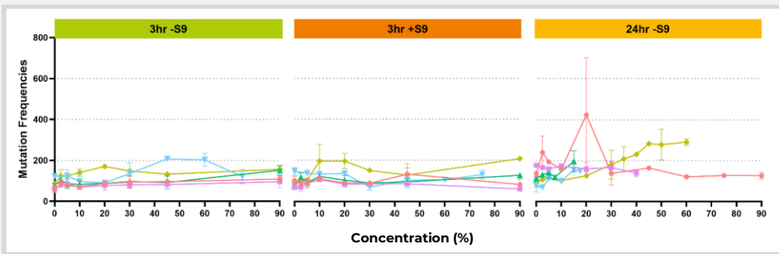


Figure 5. The mutagenicity of the test article extracts (%) was determined using MLA assay at 3 hrs -S9, 3 hrs + S9 and 24 hrs -S9. Data are mean ± SD from two culture replicates. GEF = 126 mutants per 106 viable cells.

Results: Potential signalling pathways or inflammatory response

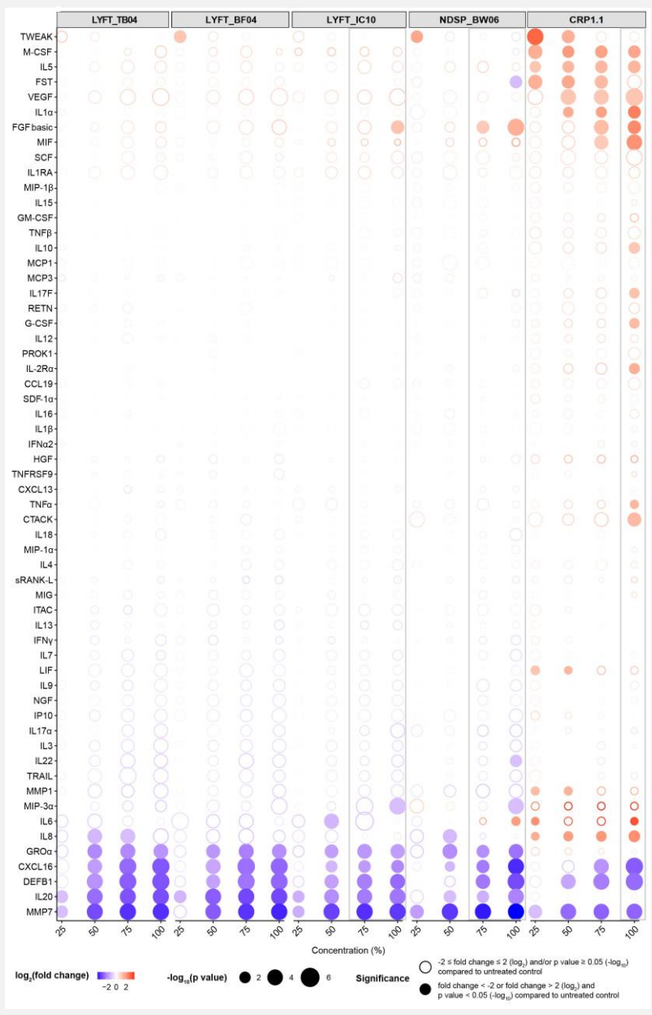


Figure 7. Inflammatory response upon exposure to five test article extracts for 48 hrs. Fold changes were calculated relative to the untreated control samples using MFI values. The grey box highlights any concentration that cause the cell viability to be less than 75% due to high cytotoxicity.

Potential signalling pathways or inflammatory response

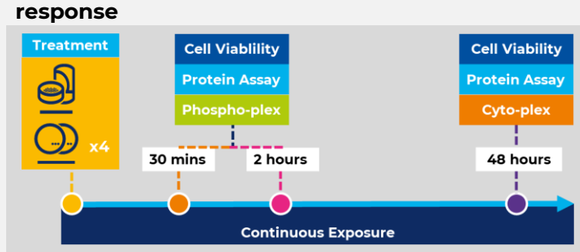


Figure 6. Experimental design and endpoints of experiments.

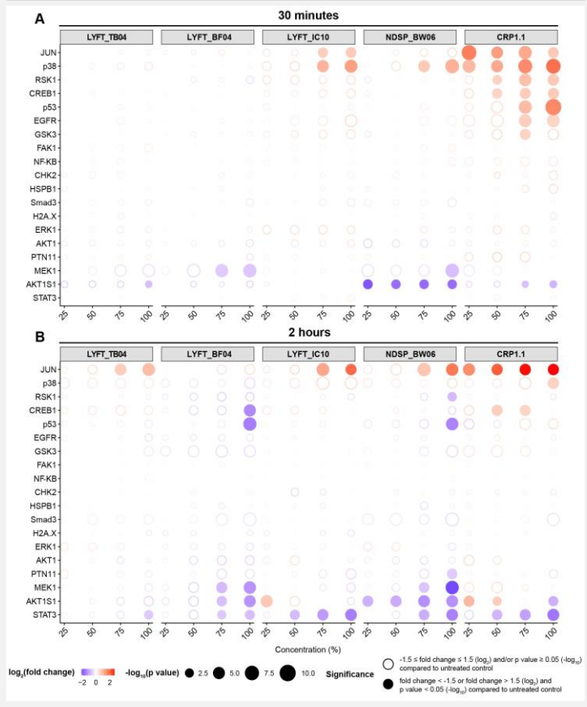
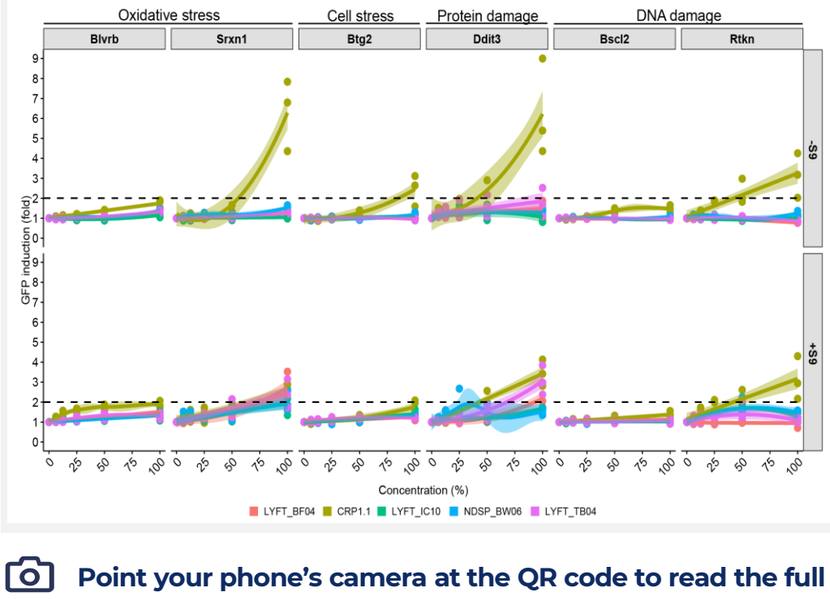


Figure 8. Phosphorylation signalling markers response measured at 30 mins and 2 hrs. Fold changes were calculated relative to the untreated control samples using median fluorescence intensities (MFI values).

Results: In vitro disease assessment: ToxTracker



In vitro disease assessment - ToxTracker

CRP1.1 induced a positive response in four toxicological endpoints in the absence of S9 and three endpoints in presence of S9 (Figure 3).

All NPs induced a positive response in Srxn1 +S9, with LYFT_BF04 and LYFT_TB04 inducing a positive response in Ddit3 +S9.

Figure 3. Genotoxic response of five test article extracts assessed with ToxTracker reporter cell lines.

Results were shown as fold induction of the six separate biomarkers: Srxn1 & Blvrb (oxidative stress), Btg2 (cellular stress), Bscl2 & Rtkn (DNA damage) and Ddit3 (protein damage). Curves show best fit and 95 % confidence interval of the fit. Dashed line shows the 2-fold change threshold in GFP induction.

Conclusion

This study demonstrated that a weight of evidence approach is required to cover a wide range of endpoints to provide sufficient in vitro data for the assessment of potential risk of NP and snus.

To this end we have used a three-step approach to analyse the biological effects of NPs and reference snus extracts in the following areas: cytotoxicity, mutagenicity/ genotoxicity, and cell signalling. In summary, NPs extracts were less biologically active in all endpoints tested, compared to snus, relevant to a range of disease processes.

Taken together with previously published data on chemical analysis and clinical studies, the data presented here contribute to the weight of evidence that suggest NPs should considered as an alternative reduced risk product.

Acknowledgements

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