NEXT GENERATION PRODUCTS

Assessment of innovative Next Generation nicotine delivery products using ToxTracker assay reveals marked reductions in genotoxicity and oxidative stress compared to cigarettes

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INTRODUCTION

Imperial Brands understands that smoking is a cause of serious 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. While science suggests that nicotine is addictive and not risk-free, Public Health experts worldwide have concluded that it is the toxicants in cigarette smoke generated by burning tobacco, and not nicotine, which is the primary cause of smoking-related disease. Tobacco Harm Reduction (THR) refers to strategies designed to reduce the health risks associated with tobacco smoking. Next Generation Products (NGP), like Heated Tobacco Products (HTP) and E-Vapour Products (EVP), deliver nicotine without burning tobacco so have the potential to play a role in THR.

There is a need for rapid and sensitive techniques to determine the biological impact of these new products to understand their tobacco harm reduction potential. Here we present data for a range of inhaled NGPs (e-vapour products: blu 2.0™, blu bar™; Heated tobacco and herbal products (HHP): Pulze 2.0 device with either iD™ or iSENZIA™ sticks, all obtained from the EU market), assessed using the ToxTracker™ assay and compared to a reference cigarette. The ToxTracker technique was extended with the use of reactive oxygen species (ROS) scavengers (N-Acetyl Cysteine (NAC) and Glutathione (GSH)), to determine if the test articles caused any indirect genotoxicity via oxidative stress in the presence of the test compounds.

METHODS

2.1 Test Articles

- 1R6F Reference Cigarette (University of Kentucky)
- Heated Tobacco Product (HTP), "Pulze" with "iD regular intense tobacco stick"
- Heated Herbal Product (HHP), "Pulze" with "iSENZIA Sunset Coral Crush stick"
- E-vapour product (EVP 1), "blu 2.0 with Golden tobacco flavour 18mg/ml nicotine"
- E-vapour product (EVP 2), "blu bar Tropical mix flavour 20mg/ml nicotine"

All NGPs were obtained from the EU market.

2.2 Smoke / Aerosol Extract Generation method

Smoke and aerosol from test products were generated with a Vitrocell VC10s (Vitrocell, Munich, Germany) smoking machine. Smoke or aerosol extracts were prepared by bubbling the sample aerosol into 3 in-line Impingers each containing 10 mL of either Phosphate Buffered Saline (PBS) solution or cell media (See Figure 1). A total stock solution of 30 mLs per test article was used.

Figure 1: Bubbling smoke/vapour exposure system

Trapped nicotine was quantified within the aerosol and smoke bubbled PBS (bPBS) samples, using liquid chromatography with tandem mass spectrometry (LC-MS/MS) with an AB Sciex API 6500 QTRAP (SCIEX, Framingham, MA, USA) using nicotine-d4 as the internal standard. For the analysis of carbonyls, bPBS samples were diluted with 2,4-dinitrophenylhydrazine (DNPH). The carbonyl-DNPH derivates were then quantified using high performance liquid chromatography with a diode-array detector (HPLC-DAD, Agilent Technologies 1100 Series).

2.3 Biological Assessment

All of the biological assessment was performed by Toxys B.V.

The ToxTracker genotoxicity assay utilises 6 green fluorescent protein (GFP) reporter cell lines measuring DNA damage, oxidative stress, p53 activation and protein damage[1]. These cell lines were exposed to the test articles for 24h±S9. Following this exposure period, the differential induction of the green fluorescent protein reporters, as well as cytotoxicity was determined using flow cytometry. Reporters with a greater than 2-fold induction of GFP fluorescence compared to controls were deemed to be a positive signal.

In an extension to the standard ToxTracker procedure, the reporter cell lines were also exposed to test compounds in the presence of NAC (10mM) or GSH (10mM)[2]. The impact of the ROS scavengers on reporter activation was determined following the 24h exposure to the compounds using flow cytometry. Potassium bromate was included as control for the ROS scavenger experiments.

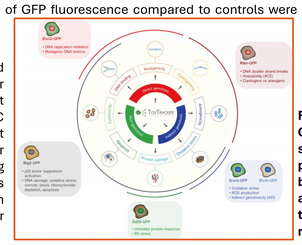


Figure 3: Overview of the stress pathways and biomarkers assessed by the ToxTracker reporter assay[1]

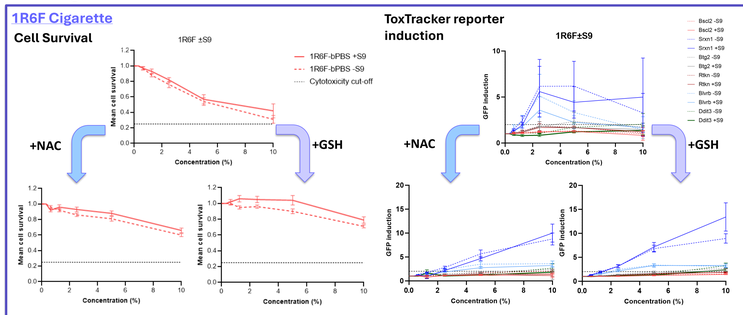
RESULTS

3.1 Dosimetry: Nicotine and carbonyl levels in bubbled PBS

- Nicotine and eight carbonyls (Formaldehyde, Acetaldehyde, Acetone, Acrolein, Propionaldehyde, Crotonaldehyde, 2-Butanone and n-Butyraldehyde) were quantified in the bPBS matrix.
- The HTP sample delivered the highest levels of nicotine to the PBS (335µg/ml), whereas the HHP delivered the lowest amount of nicotine (173µg/ml). The reference cigarette delivered 258µg/ml nicotine to the PBS, whereas the EVP products delivered 241 - 311µg/ml.
- The 1R6F bPBS samples contained the highest level of carbonyls (levels ranging from 1.5 – 150.17µg/ml). In contrast, marked reductions in carbonyls were observed for all NGP bPBS samples.

3.2 Biological Results

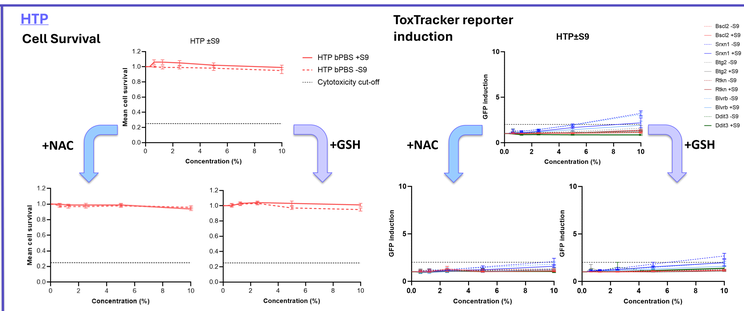
1R6F Cigarette



Significant cytotoxicity (>50%) was observed for 1R6F bPBS, which was reduced upon the addition of GSH and NAC.

The 1R6F bPBS was predicted to be genotoxic, inducing the DNA damage, oxidative stress and p53 activation markers. The ROS scavengers reduced the activation of the oxidative stress reporters, but this activation remained above the 2-fold above control positive result threshold.

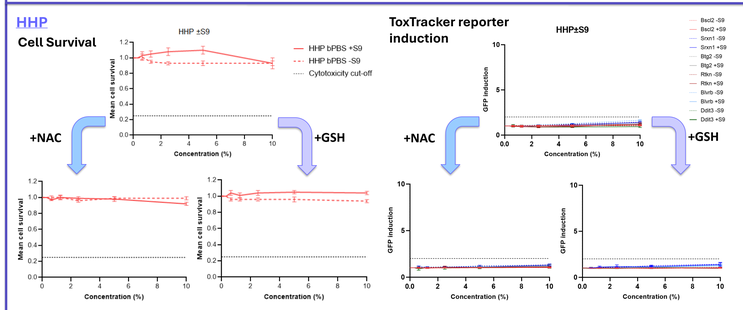
HTP



Limited cytotoxicity (±5%) was observed at the maximum tested concentration of 10 %.

The HTP bPBS did not induce a genotoxicity reporter but activated both reporters of oxidative stress (at higher concentrations compared to 1R6F). Once again, the ROS scavengers reduced the activation but not below the 2-fold threshold.

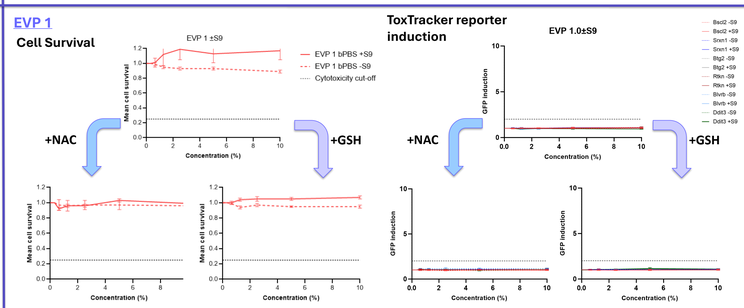
HHP



Limited cytotoxicity (±5%) was observed at the maximum tested concentration of 10 %, which was reduced upon the addition of GSH and NAC.

The HHP bPBS did not induce any genotoxicity or any other reporter, with the additional testing with ROS scavengers also having no impact.

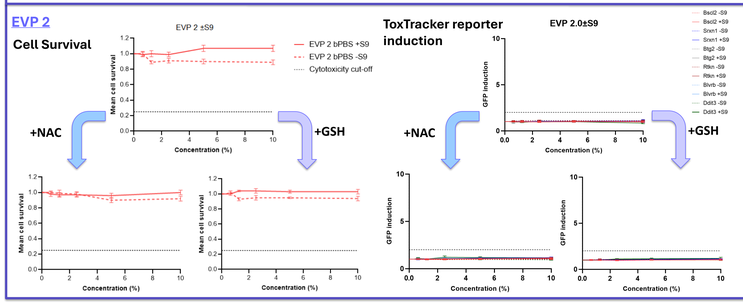
EVP 1



Limited cytotoxicity (±10%) was observed at the maximum tested concentration of 10 %, which was reduced upon the addition of GSH and NAC.

The EVP 1 bPBS did not induce any genotoxicity or any other reporter, with the additional testing with ROS scavengers also having no effect.

EVP 2



Limited cytotoxicity (±10%) was observed at the maximum tested concentration of 10 %, which was reduced upon the addition of GSH and NAC.

The EVP 2 bPBS did not induce any genotoxicity or any other reporter, with the with ROS scavengers also having no effect.

Summary Table: No-Observed Effect Level / No-Observed Genotoxic Effect Level concentrations (bPBS %) in the absence or presence of ROS scavengers

		-S9		+S9	
		NOEL	NOGEL	NOEL	NOGEL
1R6F bPBS	-	N/A	1.25%	0.625%	1.25%
	+NAC	0.625%	2.5%	1.25%	10%
	+GSH	0.625%	2.5%	0.625%	2.5%
HTP bPBS	-	2.5%	10%	2.5%	10%
	+NAC	2.5%	10%	5%	10%
	+GSH	2.5%	10%	2.5%	10%
HHP bPBS	-	5%	10%	10%	10%
	+NAC	10%	10%	10%	10%
	+GSH	10%	10%	10%	10%
EVP 1 bPBS	-	10%	10%	10%	10%
	+NAC	10%	10%	10%	10%
	+GSH	10%	10%	10%	10%
EVP 2 bPBS	-	10%	10%	10%	10%
	+NAC	10%	10%	10%	10%
	+GSH	10%	10%	10%	10%

The No Observed Effect Level (NOEL) defines the highest concentration where none of the ToxTracker reporters showed a >1.5-fold increase in fluorescence. The No Observed Genotoxicity Effect Level (NOGEL) is the highest concentration that did not activate the Bscl2-GFP and/or Rtkn-GFP reporters with a >1.5-fold increase in fluorescence.

The 1R6F bPBS was the most potent sample, inducing biological and genotoxic effects at low concentrations. In contrast none of the next generation products induced a genotoxic effect up to to the top tested concentration (10%).

CONCLUSIONS

- The Toxtracker Antioxidant assay is a rapid and sensitive technique for elucidating genotoxicological potential and subsequent mechanisms of action
- 1R6F cigarette bPBS activated the DNA damage Rtkn-GFP reporter ±S9,but not the Bscl2-GFP reporter. This sample also triggered the Btg2-GFP reporter for p53 activation, and both oxidative stress reporters ± S9. Activation of the Ddit3-GFP protein stress reporter was observed only in absence of S9. As the genotoxic signal of 1R6F cigarette smoke extract was not entirely reduced by the ROS scavengers, it is presumed that oxidative stress plays only a partial role in the observed genotoxic effect.
- The absence of a genotoxicity response from the various inhaled NGP samples adds to the growing evidence that these NGPs have tobacco harm reduction potential.

REFERENCES

[1] Czekala L, Chapman F, Simms L, Rudd K, Trelles Sticken E, Wieczorek R, Bode LM, Pani J, Moelijker N, Derr R, Brandsma I, Hendriks G, Stevenson M, Walele T. 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 May 31;36(2):129-142. doi: 10.1093/mutage/geaa033.
[2] Brandsma I, Moelijker N, Derr R, Hendriks G, Aneugen Versus Clastogen Evaluation and Oxidative Stress-Related Mode-of-Action Assessment of Genotoxic Compounds Using the ToxTracker Reporter Assay, Toxicological Sciences, Volume 177, Issue 1, September 2020, Pages 202–213, https://doi.org/10.1093/toxsci/kfaa103

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