Assessment of a Heated Tobacco Product in ToxTracker and ToxProfiler assays reveal marked reductions in biological activity compared to cigarettes

ESTIV Prague, June 3-6 2024, Abstract number: 262

Visit our Scientific Research website
www.imperialbrandscience.com

Ourania Komini[1]*, Remco Derr[2], Inger Brandsma[2], Bas ter Braak[2], Liesanne Wolters[2], Edgar Trelles Sticken[3], Roman Wieczorek[3], Sarah Jean Pour[3], Fiona Chapman[1], Liam Simms[1], Matthew Stevenson[1]

[1]Imperial Brands PLC, 121 Winterstoke Road, Bristol, BS3 2LL, UK, [2]Toxys B.V., De limes 7, 2342 DH, Oegstgeest, The Netherlands, [3]Reemtsma Cigarettenfabriken GmbH, an Imperial Brands PLC company, Albert-Einstein-Ring-7, D-22761, Hamburg, Germany
*Corresponding author's e-mail: ourania.komini@impbrands.com

1. INTRODUCTION

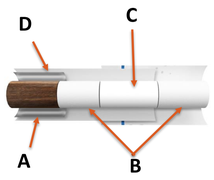
Heated Tobacco Products (HTP) are an expanding category of Next Generation Products which heat a stick of reconstituted tobacco to a defined temperature (345 °C)[1]. Rapid and mechanistically insightful *in vitro* methods are required for the biological assessment of these products. Here we explored the ToxTracker and ToxProfiler assays for the comparative assessment of a HTP (Pulze and iD sticks) to that of a cigarette (1R6F)[2,3].

2. METHODS

Test articles

- 1R6F Reference Cigarette (University of Kentucky)
- Heated Tobacco Product (HTP), "Pulze" with "iD stick" (iD Regular) (see Figure 1)

Representative image of the HTP device used in this study (reconstituted tobacco stick inserted).



Representative diagram of the HTP sticks used in this study, consisting of (A) reconstituted tobacco, (B) filters, (C) cardboard tube and (D) outer paper with aluminium foil.

Figure 1: HTP diagram

Smoke / Aerosol extract generation method

Smoke and aerosol from test products was 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 Phosphate Buffered Saline (PBS) solution (see Figure 2). A total stock solution of 30 mLs per test article was used: 1.8 puffs per mL for 1R6F cigarette and 4 puffs per mL for the HTP.

Figure 2: Bubbling smoke/vapor exposure system

Trapped nicotine and carbonyls were quantified within the aerosol and smoke bubbled PBS (bPBS) samples. Nicotine was quantified 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).

Biological assessment

Both ToxProfiler and ToxTracker assays was performed by Toxys B.V.

ToxProfiler

The ToxProfiler assay determines activation of seven specific cellular stress response pathways (oxidative stress, cell cycle stress, ER stress, autophagy, ion stress, protein stress, inflammation) utilising 7 stable genetically engineered human liver HepG2 cell lines[3] (see Figure 3). Each one of these cell lines contain a fluorescent reporter for a specific cellular stress signal transduction pathway. These cell lines were exposed to the test articles for 24h±S9. Cells were imaged using an Operetta CLS imager at 24h after treatment. The ToxProfiler assay is considered to have a positive response when a Point of Departure (PoD) is calculated.

Figure 3: Overview of the stress pathways and biomarkers covered by the ToxProfiler reporter system

ToxTracker

The ToxTracker genotoxicity assay utilises 6 green fluorescent protein (GFP) reporter cell lines measuring DNA damage, oxidative stress, p53 activation and protein damage[2]. 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.

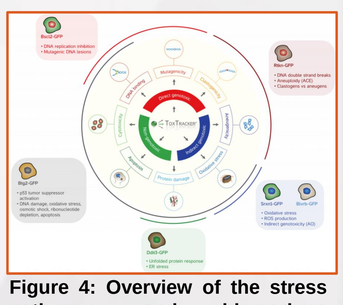


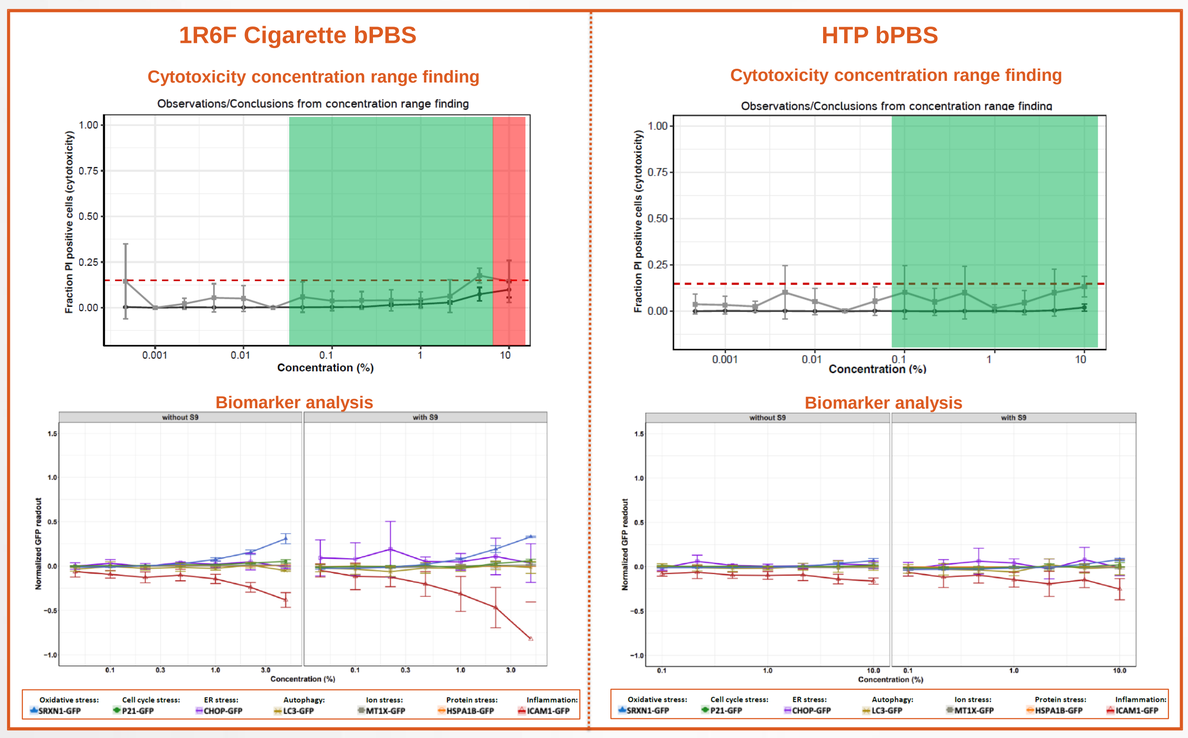
Figure 4: Overview of the stress pathways and biomarkers assessed by the ToxTracker reporter assay

3. RESULTS

Dosimetry nicotine and carbonyl levels in bubbled PBS extracts

- For each test article, PBS from all three impingers was combined to generate 30mls stock for analysis.
- Nicotine and eight carbonyls (formaldehyde, acetaldehyde, acetone, acrolein, propionaldehyde, crotonaldehyde, 2-butanone and n-butyraldehyde) were quantified in the bPBS matrix.
- The 1R6F sample delivered the highest levels of nicotine to the PBS (217µg/ml vs 166µg/ml HTP).
- The 1R6F bPBS samples contained the highest level of carbonyls (levels ranging from 1.64 – 173.09µg/ml). In contrast, the total quantified carbonyls were greatly reduced for the HTP bPBS (94%).

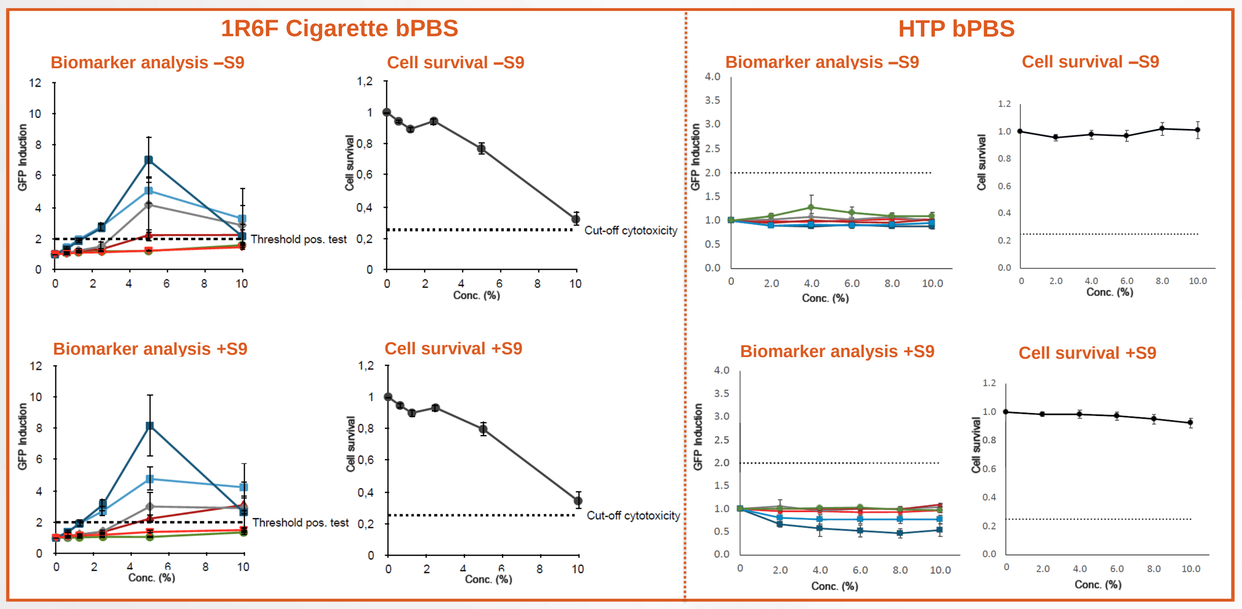
ToxProfiler results



Key – Normalized concentration response plots of cell death data of all compounds exposed in absence (black) or presence (grey) of S9 included in this project at timepoint 24h as measured with the PI stain in the parental HepG2 (wild type) line. Green shaded area: the 7 selected concentrations for the reporter assay
Red shaded area: concentrations excluded due to cytotoxicity. The red line indicates the used threshold of cytotoxicity; 15% PI positive cells.

1R6F cigarette bPBS induced an oxidative stress response in a concentration dependent manner at concentrations ≥ 0.32% without S9 and ≥ 0.23% with S9. Cell cycle stress was induced in a concentration dependent manner at concentrations ≥ 1% without S9 and ≥ 0.8% with S9.
HTP bPBS induced an oxidative stress response in a concentration dependent manner at concentrations ≥ 1% without S9 and ≥ 0.7% with S9.

ToxTracker results



The 1R6F cigarette bPBS induced cytotoxicity with increasing dose, with 50% cytotoxicity observed from 8% bPBS. A >2 fold GFP induction for Rtkn-GFP, Btg2-GFP, Srxn1-GFP and Blvrb-GFP was observed for increasing concentration of 1R6F cigarette bPBS. 1R6F cigarette bPBS did not induce either Bscl2 or Ddit3 GFPs (±S9) above the threshold. The 1R6F cigarette bPBS was classified as being genotoxic under the conditions of this test. The HTP bPBS was classified as non-genotoxic in the ToxTracker assay, because no activation of any of the reporters was observed ±S9. The impact of cytotoxicity on the levels of GFP reporter activation per test article ±S9 treatment, is shown in Table 1.

GFP reporter activation at different levels of cytotoxicity (Table 1)

Test article		Bscl2				Rtkn				Btg2				Srxn1				Blvrb				Ddit3			
		10	*25*	*50*	*75*	*10*	*25*	*50*	*75*	*10*	*25*	*50*	*75*	*10*	*25*	*50*	*75*	*10*	*25*	*50*	*75*	*10*	*25*	*50*	*75*
1R6F	*-S9*	1.1	1.3	1.4	1.4	1.2	2.2	2.2	2.2	1.1	1.8	3.9	3.3	2.1	6.1	8.1	9.0	2.6	4.8	3.9	5.0	1.2	1.3	1.5	1.6
	+S9	1.1	1.4	1.5	1.5	1.3	1.7	2.5	3.1	1.1	1.8	3.0	2.8	2.1	6.9	9.6	11.4	3.9	4.6	4.4	4.7	1.0	1.2	1.3	1.4
HTP	*-S9*	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.1	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.3	1.3	1.3	1.3
	+S9	1.0	1.0	1.0	1.0	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	0.4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Fold change induction in the six ToxTracker GFP associated endpoints at 10, 25, 50 or 75% cytotoxicity (in the presence or absence of S9; 24h exposure). Response induction above 1.5-fold, but not above the threshold of 2.0 is highlighted in paler colour text and fold changes in induction above the threshold are highlighted in darker colour text. Blank cells indicate where the level of cytotoxicity was not reached for that test article.

Summary of results

ToxProfiler

- The Point of Departure was determined for both samples, with the lowest PoD indicating the primary response
- 1R6F cigarette bPBS induced an oxidative stress response (SRXN1-GFP), at concentrations ≥ 0.32% -S9 and ≥ 0.23% +S9
- HTP bPBS also induced an oxidative stress response (SRXN1-GFP), but at higher concentration of 1% -S9 and 0.7% +S9 (3-fold higher concentrations)

ToxTracker

- 1R6F cigarette bPBS induced GFP activation with a No Observed Genotoxicity Effect Level (NOGEL) concentration of 2.5%
- HTP bPBS caused no GFP activation up to a maximum test concentration of 10% (a 4-fold higher concentration vs 1R6F cigarette bPBS)

4. CONCLUSIONS

- ToxProfiler detected the oxidative stress potential of 1R6F cigarette bPBS at low concentrations, whilst HTP bPBS induced oxidative stress at higher concentrations (3-fold higher). The ToxTracker assay determined that 1R6F cigarette bPBS was genotoxic, whereas HTP bPBS was non-genotoxic up to the maximum tested concentration of 10% bPBS
- Effect of liver metabolism was also assessed by running ToxTracker and ToxProfiler assays in the presence of S9. Liver metabolism did not have an effect on the ToxProfiler and ToxTracker results for these compounds
- The reduced biological activity of HTP aerosol relative to cigarette smoke, using bPBS extracts, in both the ToxTracker and ToxProfiler assays add to the growing evidence that these products have harm reduction potential.
- Both techniques provided rapid, sensitive detection of mechanistic effects of these complex mixtures and should be considered for future assessment strategies of Next Generation Products.

REFERENCES

1. Chapman F, Sticken ET, Wieczorek R, Pour SJ, Dethloff O, Budde J, Rudd K, Mason E, Czekala L, Yu F, Simms L, Nahde T, O'Connell G, Stevenson M. Multiple endpoint in vitro toxicity assessment of a prototype heated tobacco product indicates substantially reduced effects compared to those of combustible cigarette. Toxicol In Vitro. 2023 Feb;86:105510. doi: 10.1016/j.tiv.2022.105510.
2. 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.
3. Wink S, Hiemstra S, Herpers B, van de Water B. High-content imaging-based BAC-GFP toxicity pathway reporters to assess chemical adversity liabilities. Arch Toxicol. 2017 Mar;91(3):1367-1383. doi: 10.1007/s00204-016-1781-0. Epub 2016 Jun 29. PMID: 27358234; PMCID: PMC5316409.