NEXT GENERATION PRODUCTS

# Assessment of a range of Next Generation Nicotine products using ToxTracker® and ToxProfiler™ MAX assays reveal marked reductions in biological activity compared to a cigarette

EUROTOX | 14-17th September 2025 | P21-01 Poster Viewing II (PV02)

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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 is the primary cause of smoking-related disease, not nicotine.

Tobacco Harm Reduction (THR) refers to strategies designed to reduce the health risks associated with tobacco smoking. Next Generation Nicotine Products (NGP) are an emerging category of smoke-free products, which deliver nicotine to the adult user, but crucially with marked reductions of toxicants when compared to cigarettes.

These highly innovative products require an equally innovative, contemporary toxicological assessment approach to sensitively and quickly assess their biological activity[1].

Here we used the ToxTracker® and ToxProfiler™ MAX assays to compare biological responses from a cigarette (1R6F Reference cigarette) and a selection of inhaled NGP (E-Vapour (EVP), Heated tobacco (HTP) and Heated herbal (HHP) products).

ToxTracker is a mechanistic mammalian-cell based genotoxicity assay, whereas ToxProfiler MAX is a human cell-based assay that can quantify cellular stress.

## METHODS

**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 of the NGP were obtained from the EU market.

**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.

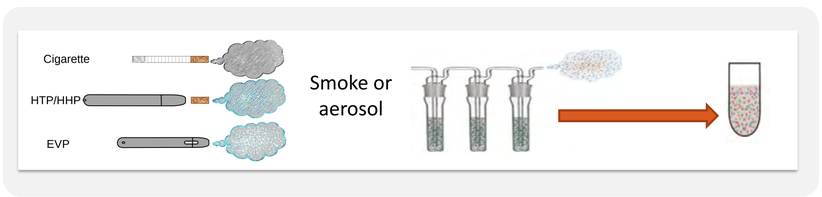



**Figure 1: Bubbling smoke/vapour exposure system**

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).

**Biological Assessment**

All 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[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.

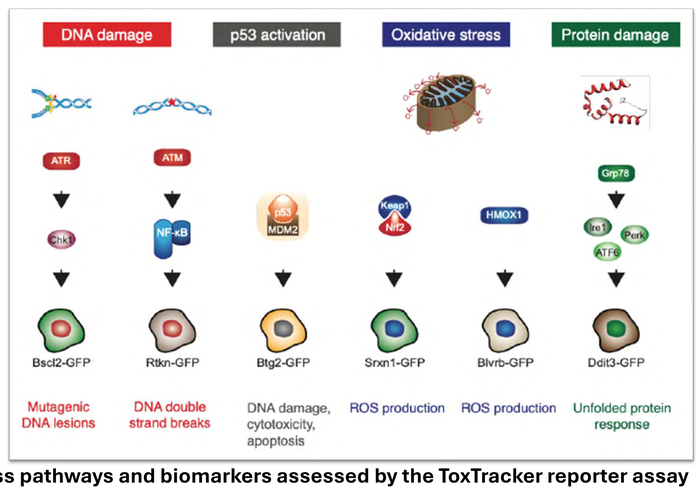
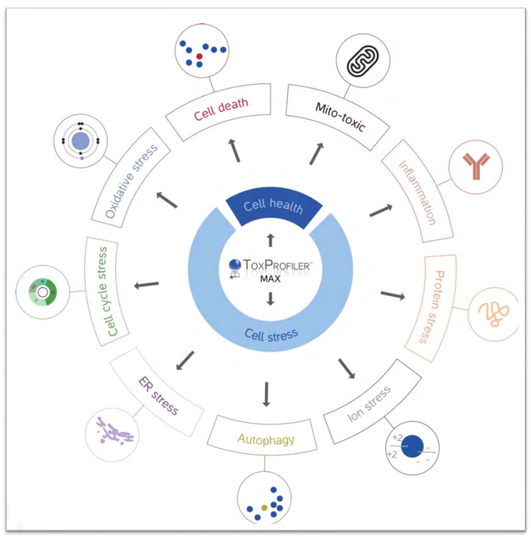



**Figure 2→:**
**Overview of the stress pathways and biomarkers assessed by the ToxTracker reporter assay**




The ToxProfiler platform consists of a panel of seven reporter cell lines cultured as a 2D monolayer, in which biomarkers of several of such cell stress response pathways have been tagged with a fluorophore (GFP)[3]. With this platform we can dynamically observe activation of these stress pathways (upon chemical treatment) using a high throughput confocal imaging system. For the extended ToxProfiler MAX assay also the glu/gal assay is integrated for which the cytotoxicity is quantified in glucose and galactose conditions. The lack of glucose will force the HepG2 cells to switch their metabolism from a glucose-dependent glycolysis to a mitochondria-dependent oxidative phosphorylation. In case this switch causes significantly more cytotoxicity, it is an indication that the test substance is a mitochondrial toxicant.

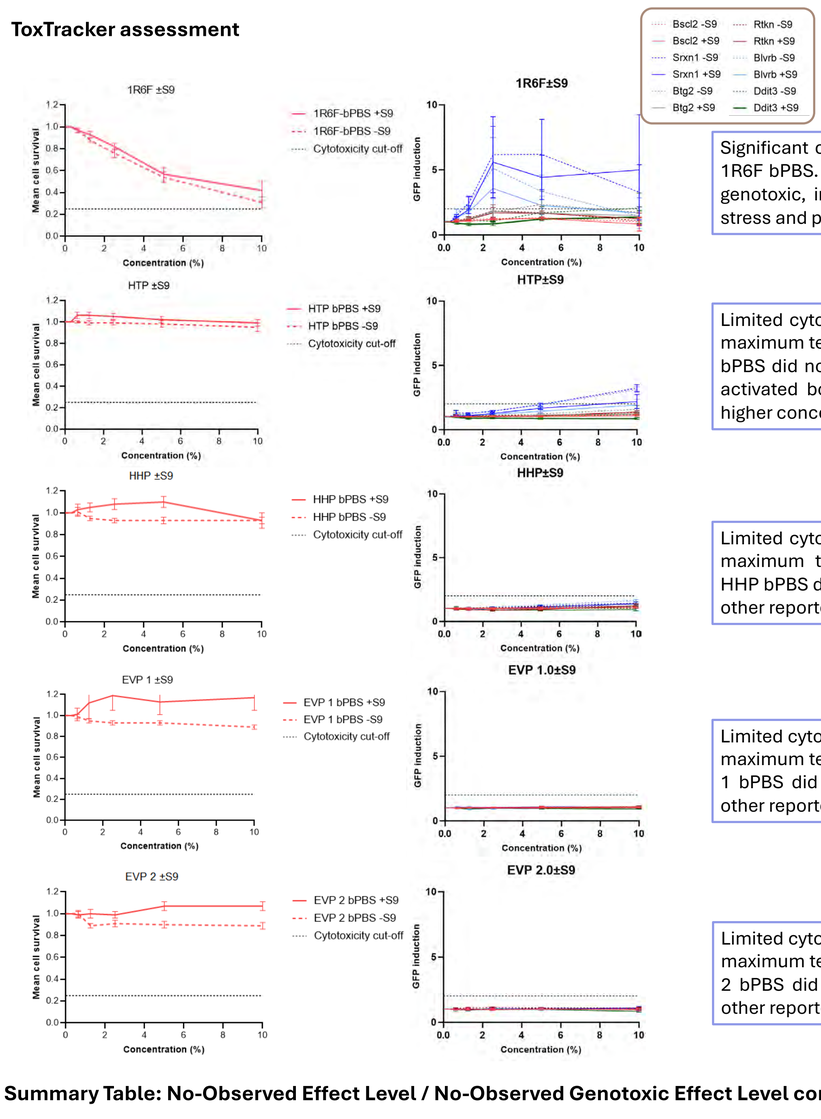
**←Figure 3:**
**Overview of the stress pathways and biomarkers covered by the ToxProfiler MAX reporter system**

## RESULTS

**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 – 150µg/ml). In contrast, marked reductions in carbonyls were observed for all NGP bPBS samples.

**ToxTracker assessment**




Significant cytotoxicity (>50%) was observed for 1R6F bPBS. The 1R6F bPBS was predicted to be genotoxic, inducing the DNA damage, oxidative stress and p53 activation markers.

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).

Limited cytotoxicity (±5%) was observed at the maximum tested concentration of 10 %. The HHP bPBS did not induce any genotoxicity or any other reporter.

Limited cytotoxicity (±10%) was observed at the maximum tested concentration of 10 %. The EVP 1 bPBS did not induce any genotoxicity or any other reporter.

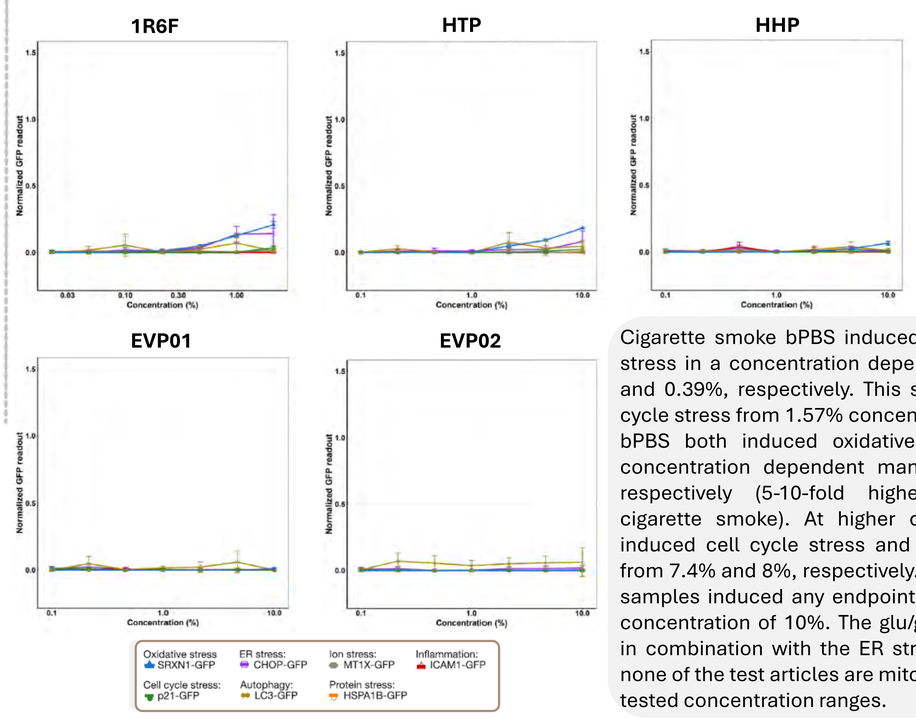
Limited cytotoxicity (±10%) was observed at the maximum tested concentration of 10 %. The EVP 2 bPBS did not induce any genotoxicity or any other reporter.

**Summary Table: No-Observed Effect Level / No-Observed Genotoxic Effect Level concentrations (bPBS %)**

| | -S9 | | +S9 | |
|---|---|---|---|---|
| | NOEL | NOGEL | NOEL | NOGEL |
| 1R6F | N/A | 1.25% | 0.625% | 1.25% |
| HTP | 2.5% | 10% | 2.5% | 10% |
| HHP | 5% | 10% | 10% | 10% |
| EVP1 | 10% | 10% | 10% | 10% |
| EVP2 | 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 the top tested concentration (10%).

**ToxProfiler MAX assessment**




Cigarette smoke bPBS induced oxidative stress and ER stress in a concentration dependent manner from 0.3% and 0.39%, respectively. This sample also induced cell cycle stress from 1.57% concentration. The HTP and HHP bPBS both induced oxidative stress responses in a concentration dependent manner from 1.5% and 3% respectively (5-10-fold higher concentrations than cigarette smoke). At higher concentrations HTP also induced cell cycle stress and ER stress was observed from 7.4% and 8%, respectively. Notably, none of the EVP samples induced any endpoint up to the maximum test concentration of 10%. The glu/gal medium switch assay in combination with the ER stress results revealed that none of the test articles are mitochondrial toxicants in the tested concentration ranges.

## CONCLUSIONS

- Both ToxTracker and ToxProfiler techniques, sensitively detected oxidative stress responses from the cigarette smoke extract. Both HTP and HHP aerosol extracts displayed oxidative stress responses albeit at 5-10-fold higher concentrations than cigarette smoke extracts. None of the EVP samples induced any oxidative stress.
- The 1R6F cigarette bPBS activated the DNA damage Rtkn-GFP reporter, but not the Bscl2-GFP reporter. This sample also triggered the Btg2-GFP reporter for p53 activation.
- None of the NGP extracts induced a genotoxic effect up to the highest test concentration.
- Likewise, none of the test samples displayed mitochondrial toxicity, at the tested concentrations.
- The reduced biological activity of NGP aerosol extracts relative to cigarette smoke extracts, in both the ToxTracker and ToxProfiler MAX assays add to the growing evidence that these products have tobacco harm reduction potential.

## REFERENCES

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