Next generation products predicted to have no potential to induce developmental toxicity in the devTOX *quick*Predict assay

British Toxicology Society **April 15th – 17th 2019**

IMPERIAL BRANDS

SCIENCE

Visit our Scientific Research website
www.imperialbrandscience.com

Victoria Taverner[1], Jessica Palmer[2], Liam Simms[1], Roman Wieczorek[3], Lisa Maria Bode[3], Matthew Stevenson[1]

1. Imperial Brands PLC, 121 Winterstoke Road, Bristol, BS3 2LL, UK
2. Stemina Biomarker Discovery Inc, 504 South Rosa Road, Suite 150 Madison, WI 53719
3. Reemtsma Cigarettenfabriken GmbH, An Imperial Brands PLC Company, Albert-Einstein-Ring-7, D-22761, Hamburg, Germany

1. INTRODUCTION / OBJECTIVES

A range of tobacco-based and tobacco-free next generation products (NGPs) are commercially available and there is a growing scientific consensus that NGPs may present a less harmful alternative to cigarettes for adult smokers. With the increased popularity of NGPs amongst adult smokers it is important that their potential biological effects can be assessed quickly and accurately. Although not recommended for use by pregnant women, in order to address regulatory concerns, further research is required to assess whether NGP aerosols present a developmental risk to the unborn foetus. The objective of this study was to compare the potential developmental toxicity of NGP aerosols to cigarette smoke using Stemina's devTOX *quick*Predict (devTOX^{qP}) human pluripotent stem cell-based assay. The devTOX^{qP} assay is a high-throughput *in vitro* developmental toxicity assay used to signal whether a test compound has the potential to cause developmental toxicity in humans. Ornithine and cystine are both involved in metabolic pathways important for normal cell proliferation and differentiation during development. Changes in the ratio of ornithine/cystine levels have been experimentally associated with common mechanisms of developmental toxicity (Palmer *et al*., 2013). The devTOX *quick*Predict assay uses the metabolic perturbation of the biomarkers ornithine/cystine ratio (o/c ratio) to predict whether a test compound exhibits developmental toxicity potential.

2. MATERIALS AND METHODS

2.0 Test Samples

- 3R4F Kentucky Reference Cigarette
- Tobacco Heated Product (THP)
- Hybrid Product (HYB); 1.8 % nicotine
- *my*blu™ Tobacco flavour; 1.6% nicotine

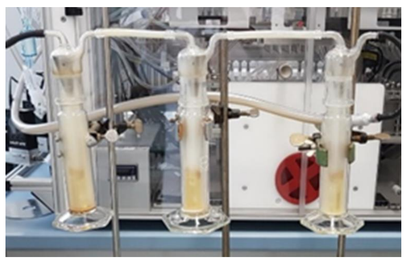

Figure 1: Bubbling smoke/aerosol exposure system

2.1 Smoke / Aerosol Extract Generation Method

- Aerosol from test products was generated with a Vitrocell VC10s (Vitrocell, Munich, Germany) smoking machine.
- The 3R4F smoke and the THP aerosol were generated using the ISO intense smoking regime, with HYB and *my*blu™ vaped according to CORESTA Recommended Method N° 81.
- Smoke or aerosol extracts were prepared by bubbling the sample aerosol into 3 in-line impingers each containing 10 mL Phosphate Buffered Saline (See Figure 1).
- A total stock solution of 30 mls per test article was used: 1.8 puffs per ml for 3R4F and 4 puffs per ml for NGPs.
- Nicotine and carbonyls trapped in fresh PBS samples were quantified using an LC-MS/MS and HPLC-DAD method respectively.

2.2 devTOX *quick*Predict Assay Method

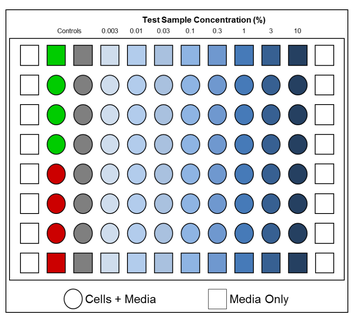

Figure 2: Test plate layout. iPS cells were plated in the inner 60 wells of a 96-well plate (filled circles). Each 96-well plate included reference (0.1% DMSO, 6 wells, **grey circles**), positive (1 µM Methotrexate, 3 wells, **red circles**), and negative (0.005 µM Methotrexate, 3 wells, **green circles**) control treatments, as well as eight concentrations of two test articles (0.003-10%, 3 wells/concentration, **blue circles**). Media controls (lacking cells, ± test article) were also included for each treatment to assess the impact of the test article on the sample matrix (filled squares).

- Human induced pluripotent stem (iPS) cells (Cell Line: HYR0103; ATCC, Manassas, VA, USA) were maintained in the undifferentiated state in mTeSR1 (StemCell Technologies, Vancouver, BC, Canada) on Matrigel (Corning, Bedford, MA, USA).
- iPS cells were exposed to eight concentrations (0.003-10%) of each test sample for 48 hours, with media and test sample replacement every 24 hours. Each experimental plate contained a reference control (0.1% DMSO), positive control (1 µM Methotrexate), and negative control (0.005 µM Methotrexate) to ensure the hPSC metabolism met the assay specifications (Figure 2).
- Spent media from the last 24-hour treatment period was collected and cell viability was assessed using the CellTiter-Fluor Cell Viability Assay (Promega, Madison, WI, USA).
- Ornithine and cystine were measured using liquid chromatography-high resolution mass spectrometry (LC-HRMS).
- Relative fold changes for each metabolite were calculated by normalizing the response of each treatment to the reference sample. The o/c ratio was determined by dividing the reference-normalized value for ornithine by the reference-normalized value for cystine.
- GraphPad Prism v 8.0 (GraphPad Software, San Diego, CA, USA) was used to fit non-linear dose-response curves and determine the developmental toxicity potential (dTP, o/c ratio) and toxicity potential (TP, cell viability) concentrations (Figure 3).
- An extra sum-of-squares *F* test was used to determine if the dose-response curve for the 3R4F sample was significantly different from the other three test samples (THP, HYB, *my*blu™).

N	Accuracy	Sensitivity	Specificity
112	86%	84%	87%

Table 1: devTOX^{qP} Accurately Predicted Developmental Toxicity of a Broad Range of Compounds

The devTOX^{qP} has excellent accuracy, specificity and good sensitivity with a wide range of chemotypes. The tested chemicals included pharmaceuticals, agrichemicals, cosmetic ingredients, industrial solvents, and environmental chemicals (Palmer., 2019).

3. RESULTS / DISCUSSION

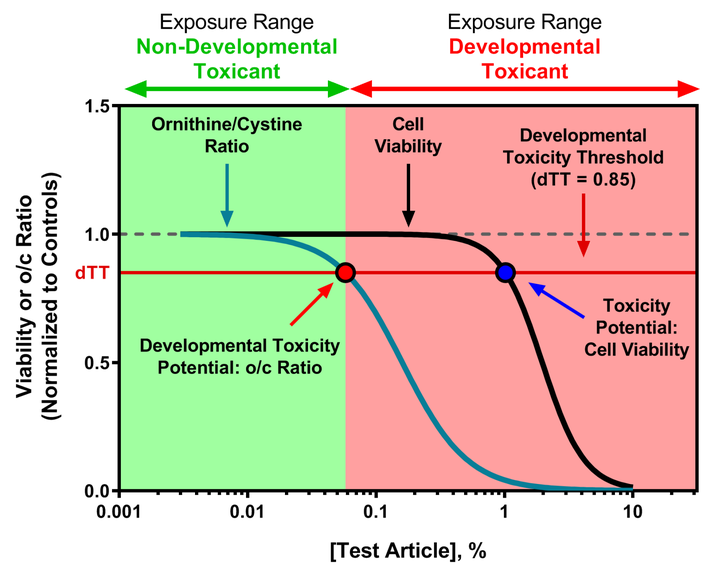

Figure 3: devTOX^{qP} Results Interpretation.

The point where the o/c ratio dose-response curve (teal line) crosses the developmental toxicity threshold (dTT; red line) indicates the exposure level where a test sample has developmental toxicity potential (red point).

The cell viability toxicity potential (blue point) is the exposure level where the cell viability dose-response curve (black line) exceeds the dTT.

Described in Palmer *et al*., 2013.

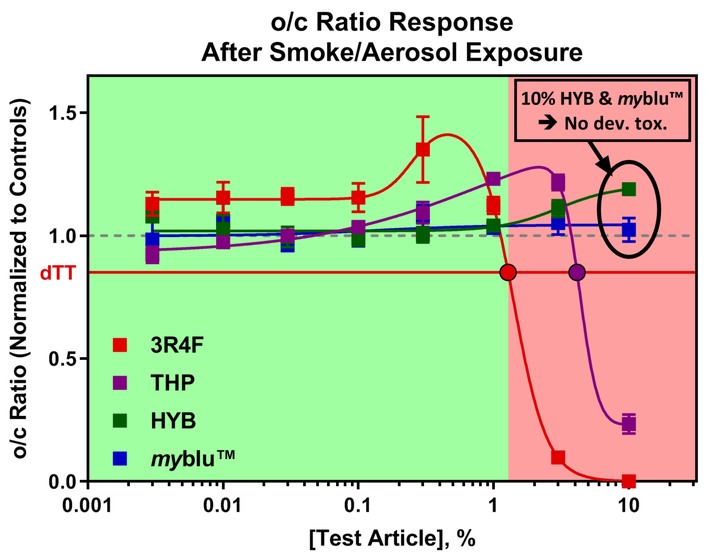

Figure 4: The o/c ratio dose-response curves for 3R4F and THP extracts crossed the dTT at 1.3% and 4.3%, respectively. This indicates that these samples are predicted to have the potential to cause developmental toxicity *in vivo* at or above their respective dTP concentration. The increase in the o/c ratio observed prior to cell death is due to increased cystine uptake as an adaptive response to oxidative stress.

HYB and *my*blu™ extracts did not elicit a metabolic response, indicating that these test samples are predicted to have no potential for developmental toxicity *in vivo* at the concentrations tested.

Figure 5: 3R4F and THP extracts decreased human iPS cell viability at concentrations similar to where a decrease in the o/c ratio was observed (1.1% and 4.1%, respectively). The decrease in cell viability indicates that these samples may cause cell death in the developing embryo.

HYB and *my*blu™ did not decrease human iPS cell viability at the concentrations tested.

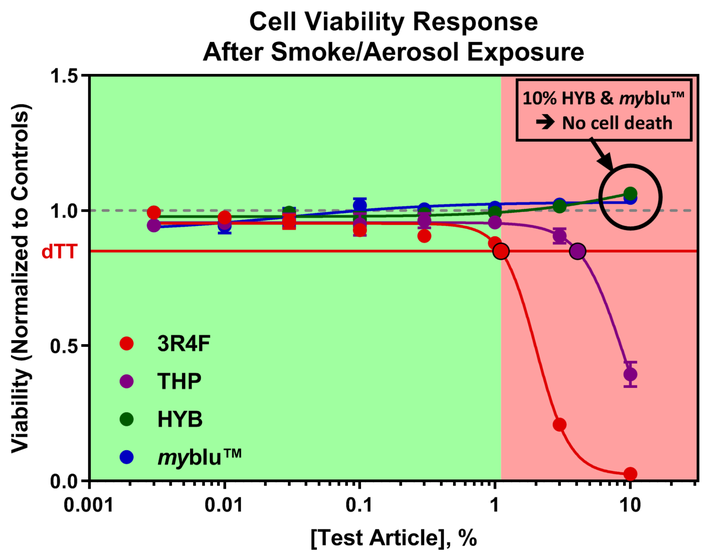

Table 2: Test Sample Concentrations with a Statistically Significantly Different o/c Ratio or Cell Viability Response from 3R4F (*p*<0.01).

Endpoint	THP	HYB	*my*blu™
o/c Ratio	0.3%, ≥3%	0.3%, ≥3%	0.3%, ≥3%
Cell Viability	≥3%	≥0.3%	≥0.1%

A two-way ANOVA followed by Fisher's Least Significant Difference *post hoc* test was used to determine if the response for 3R4F was significantly different from the other three test samples. *P* values were adjusted for multiple comparisons using Benjamini and Hochberg's method to control the false discovery rate.

4. CONCLUSIONS

- The dose-response curves for THP, HYB, and *my*blu™ were all statistically significantly different from 3R4F (extra-sum-of-square *F* test, *p*<0.0001) for both cell viability and o/c ratio.
- THP induced significantly less cytotoxicity and exhibited a significantly weaker metabolic perturbation in the o/c ratio compared to 3R4F (*p*<0.001).
- Both *my*blu™ and HYB induced no cytotoxicity under the conditions of test and showed no response in the o/c ratio predicting no potential for developmental toxicity *in vivo*.
- Although the devTOX^{qP} assay shows promise at predicting developmental toxicity, as with other *in vitro* models, it cannot fully reproduce all events contributing to the disruption of normal human developmental by exogenous chemicals. However, the devTOX^{qP} assay has utility as part of a weight of evidence approach for the assessment of NGPs.

REFERENCES

1. Palmer JA, Smith AM, Egnash LA, Conard KR, West PR, Burrier RE, Donley EL, Kirchner FR. Establishment and assessment of a new human embryonic stem cell-based biomarker assay for developmental toxicity screening. Birth Defects Res B Dev Reprod Toxicol. 2013; 98(4)343-363
2. Palmer, JA. Evaluation of the Reference Chemicals Suggested in the Draft ICH S5(R3) Guideline with a Human Pluripotent Stem Cell-Based Developmental Toxicity Assay. In: The Toxicologist: Supplement to Toxicological Sciences, 168 (1), Society of Toxicology, 2019. Abstract no. 3203.