An Overview of Toxicity Testing in 21st Century (TT21C) *In Vitro* Methods to Assess Next Generation Products

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1. INTRODUCTION

Combustible cigarettes are known to cause serious disease in smokers, including lung cancer, heart disease and emphysema. A range of next generation products (NGPs), which do not involve combustion or do not contain tobacco, are commercially available and there is a growing understanding that these NGPs may be a less harmful alternative to combustible cigarettes. TT21C methodologies can be used to assess the harm reduction potential of NGPs using human-derived cellular systems and biological responses. The harm reduction potential of three different NGPs were compared to the reference cigarette (3R4F) in a series of *in vitro* assays. Phosphate buffered saline (PBS) extracts were used within *in vitro* assays where direct exposure to smoke/aerosol was not possible.

2. SAMPLE GENERATION AND QUANTIFICATION

2.1 Test Articles

- Kentucky 3R4F Reference Cigarette
- Commercially available heated tobacco product (THP)
- Commercially available hybrid product (HYP)
- E-vapour product: *my*blu™ device and pod (1.6% [w/w] nicotine; tobacco flavour)

2.2 Smoke and Aerosol Extract Generation

Smoke and aerosol was generated using the following regimes:

- 3R4F smoke and THP aerosols were generated using ISO Intense regime 20778 (55ml puff volume, 2 s puff duration, 30 s puff interval).
- HYB and myblu aerosols were generated using CORESTA Recommended Method N°81 (55ml puff volume, 3 s puff duration, 30 s puff interval).

Smoke and aerosols 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 PBS. A total stock solution of 30mls per test article was used: 1.8 puffs per ml for 3R4F (total 54 puffs) and 4 puffs per ml for NGPs (total 120 puffs).

Nicotine and carbonyls trapped in fresh bubbled PBS samples were quantified using LC-MS/MS and LC-DAD methods respectively. For nicotine measurement, the internal standard nicotine-d4 was used. For carbonyl determination DNPH (2,4-Dinitrophenylhydrazine) was used and the carbonyl-DNPH derivatives were measured.

Quantification of nicotine and carbonyls in aerosol or smoke bubbled PBS (bPBS) extracts are shown in *Table 1.*

Table 1: *Nicotine and carbonyl quantification of PBS extracts*

Analyte level (µg/ml)	3R4F	THP	HYB	myblu	LOQ
Nicotine	82.5	123.0	53.0	152.0	0.01
Formaldehyde	5.9	0.9	1.0	<LOQ	0.25
Acetaldehyde	157.1	52.9	<LOQ	<LOQ	1.5
Acetone	24.0	5.4	<LOQ	<LOQ	1.0
Acrolein	9.4	1.3	<LOQ	<LOQ	0.5
Propionaldehyde	9.5	3.5	<LOQ	<LOQ	0.5
Crotonaldehyde	6.2	0.6	<LOQ	<LOQ	0.5
2- Butanone (MEK)	6.3	1.3	<LOQ	<LOQ	0.5
n-Butyraldehyde	3.6	3.6	<LOQ	<LOQ	0.5

3. *IN VITRO* TOXICITY ASSAYS

3.1 Regulatory toxicity

The following regulatory *in vitro* assays were performed on the PBS extracts in accordance with ISO 17025: Neutral red uptake (NRU) in HepG2 and BEAS-2B cells in compliance with in compliance with OECD TG 129; Ames assay in TA98 (+S9) and TA100 (+S9) in compliance with OECD TG 471; and *in vitro* micronucleus (IVM) with V79 (3hrs +/- S9, 24hrs – S9) in compliance with OECD TG 487.

- There was a clear cytotoxicity effect for 3R4F, a reduced effect for THP and no effect for myblu and HYB under the test conditions (n=3).
- There was marked mutagenicity for 3R4F with TA100+S9 which was reduced for THP (~3 times lower), weak for HYB and no effect was observed for myblu (n=3) under the test conditions. With TA98+S9 a mutagenic response was observed for 3R4F only.
- None of the bPBS extracts elicited any effects in the IVM assay.

3.2 Cellular Transformation Assay (CTA)

The Bhas 42 CTA assay was conducted on the PBS extracts by BioReliance according to OECD draft Guidelines (2016).[1]

- 3R4F showed extensive cytotoxicity at concentrations >1%. THP, HYB and myblu demonstrated little/no cytotoxic effect at the highest concentration (5%) (*Figure 1*).
- Only 3R4F was positive for promoting activity (*p<0.05 [ANOVA, Dunnett's post-hoc]; statistically significant increase) (*Figure 1*).

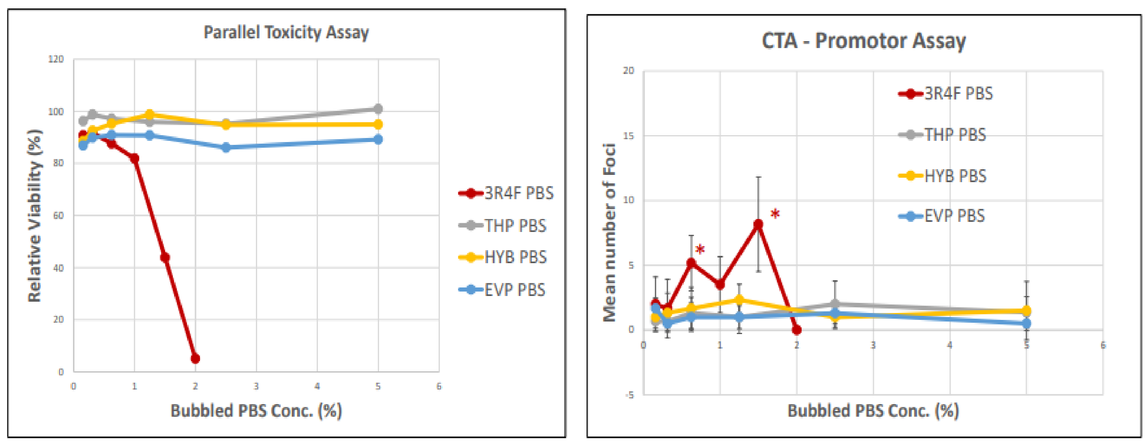

Figure 1*: Cytotoxicity and promotion activity of PBS extracts in Bhas 42 CTA assay.*

3.3 Endothelial cell migration

Human umbilical vein endothelial cells (HUVECs) were exposed to up to 10% PBS extracts for 30 hours and scratch wounded using methodology previously described by Rudd *et al.*[2] The RWD50 (50% relative wound density) is defined as the time point at which 50% of the initial scratch wound area is occupied by migrated cells. RWD50 plotted against the concentration of bPBS extract followed a linear model and is defined as cRWD50

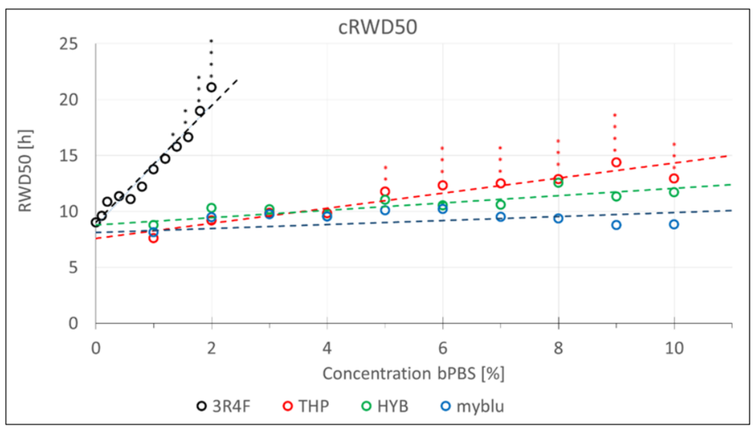

Figure 2*: cRWD50. Image acquisition was performed every 2h for 30h.*
*Key to significance: * p ≤ 0.05; ** p ≤ 0.01 *** p ≤ 0.005 **** p ≤ 0.0001*

- 3R4F displayed significant inhibitory activity on HUVEC migration at concentrations >1.4% (p ≤ 0.05), resulting in a significant increase in cRWD50 after treatment (*Figure 2*).
- THP and HYB extracts showed lower migration activity over control indicating slight inhibition of the wound healing activity. A Dunnett's test with each individual concentration confirmed the effects of THP only; at PBS concentrations >5% (p ≤ 0.01).
- The myblu extract did not show any significant inhibition up to a maximum tested concentration of 10%.

3.4 Reproductive Toxicity Screening

- The devTOX quickPredict assay was conducted by Stemina Biomarker Discovery, Inc. using methods described by Palmer *et al.*[3] Human induced pluripotent stem 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.
- 1% 3R4F and 4% THP had a significant effect on cytotoxicity (data not shown) and ornithine/ cystine (O/C) ratio indicating potential reproductive effect due to cytotoxicity (*Figure 3*). THP induced significantly less cytotoxicity (data not shown) and exhibited a significantly weaker metabolic perturbation in the o/c ratio compared to 3R4F (P<0.001 [extra-sum-of-square *F* test])
- No reproductive effect was observed at any tested concentration for HYB and myblu (*Figure 3*).
- The dose-response curves for THP, HYB, and myblu were all statistically significantly different from 3R4F (p<0.0001 [extra-sum-of-square *F* test]) for both cell viability and o/c ratio.

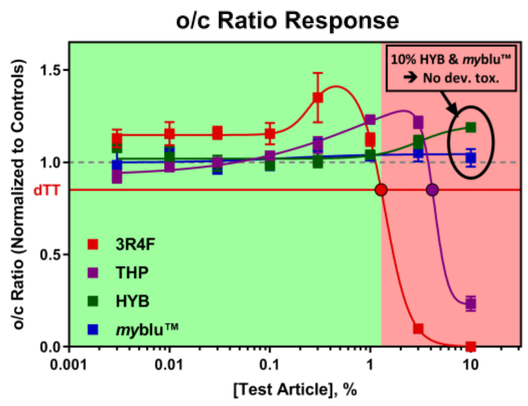

Figure 3: *The o/c ratio dose-response curves for 3R4F, THP, HYB and myblu extracts*

3.5 High Content Screening

- Human normal bronchial epithelial cells (NHBEs) from a single donor were treated with PBS extracts for 2, 4 and 24 hours, using methods previously described by Trelles-Sticken *et al*.[4] A summary of results is shown in *Table 2*.

Table 2*: The concentration of PBS extract (%) for the test articles that elicits a response in multiple cellular endpoints, measured using high content screening. '-' indicates no response was observed.*

	2 hour treatment	4 hour treatment				24 hour treatment			
	GSH	Cytotox	GH2AX	p-c-jun	NfKB	Cytotox	GH2AX	p-c-jun	NfKB
3R4F	2-5%	-	3-4%	2.5-4%	4%	1-4%	3-4%	4%	4%
THP	-	-	-	6%	4.5-10%	-	9-10%	-	9-10%
HYB	-	-	-	-	-	-	-	-	-
myblu	-	-	-	-	-	-	-	-	-

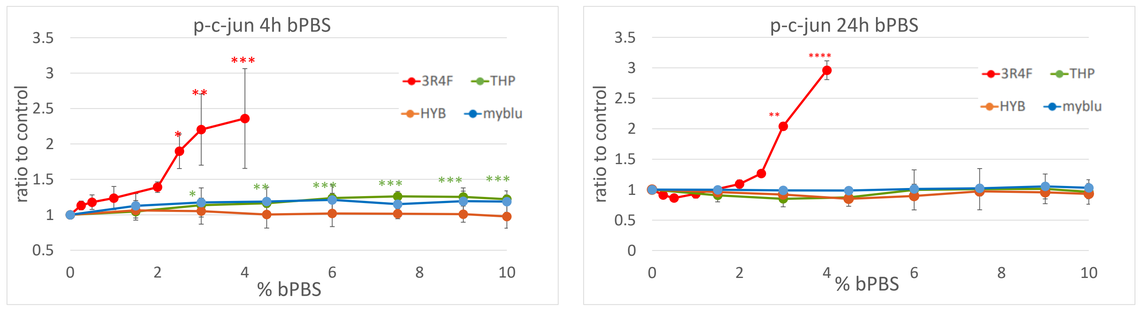

Figure 4: *p-c-jun immunostaining after 4 and 24 hour of PBS exposure from different test article extracts*
*Key to significance: * p ≤ 0.05; ** p ≤ 0.01 *** p ≤ 0.005 **** p ≤ 0.0001*

- Low concentrations of 3R4F extract triggered different cellular endpoints (oxidative stress, translocation of NfkB, phosphorylation of c-jun, and phosphorylation of histone H2AX) that resulted in statistically significantly (P<0.05 [ANOVA, Dunnett's post-hoc] reduced cell counts following 24h of exposure.
- Cells treated with the THP extract showed increased phosphorylation of c-jun *(Figure 4*), translocation of NfkB and phosphorylation of histone H2AX following 4h or 24h treatment, at higher concentrations than for 3R4F *(Table 2).*
- No statistically significant effects were observed with up to 10% PBS from HYB and myblu extracts

4. CONCLUSIONS

- This study demonstrates the suitability of aerosol bubbled PBS as an exposure media in established *in vitro* assays.
- The generated results can be used as part of a weight of evidence approach to substantiate the harm reduction potential for NGPs for adult smokers. Under the study conditions, the ranking of harm for the products could be defined as 3R4F>THP>HYB>myblu. It should be noted that large differences in biological response were observed between 3R4F and THP under the test conditions.
- Further studies, including clinical studies, are required to validate the findings in the presented work and to establish the ranking of potential harm.

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