THE APPLICATION OF IN VITRO TOXICITY TESTING IN 21ST CENTURY (TT21C) FOR NEXT GENERATION PRODUCTS

Liam Simms & Edgar Trelles Sticken | CORESTA | Hamburg 2019

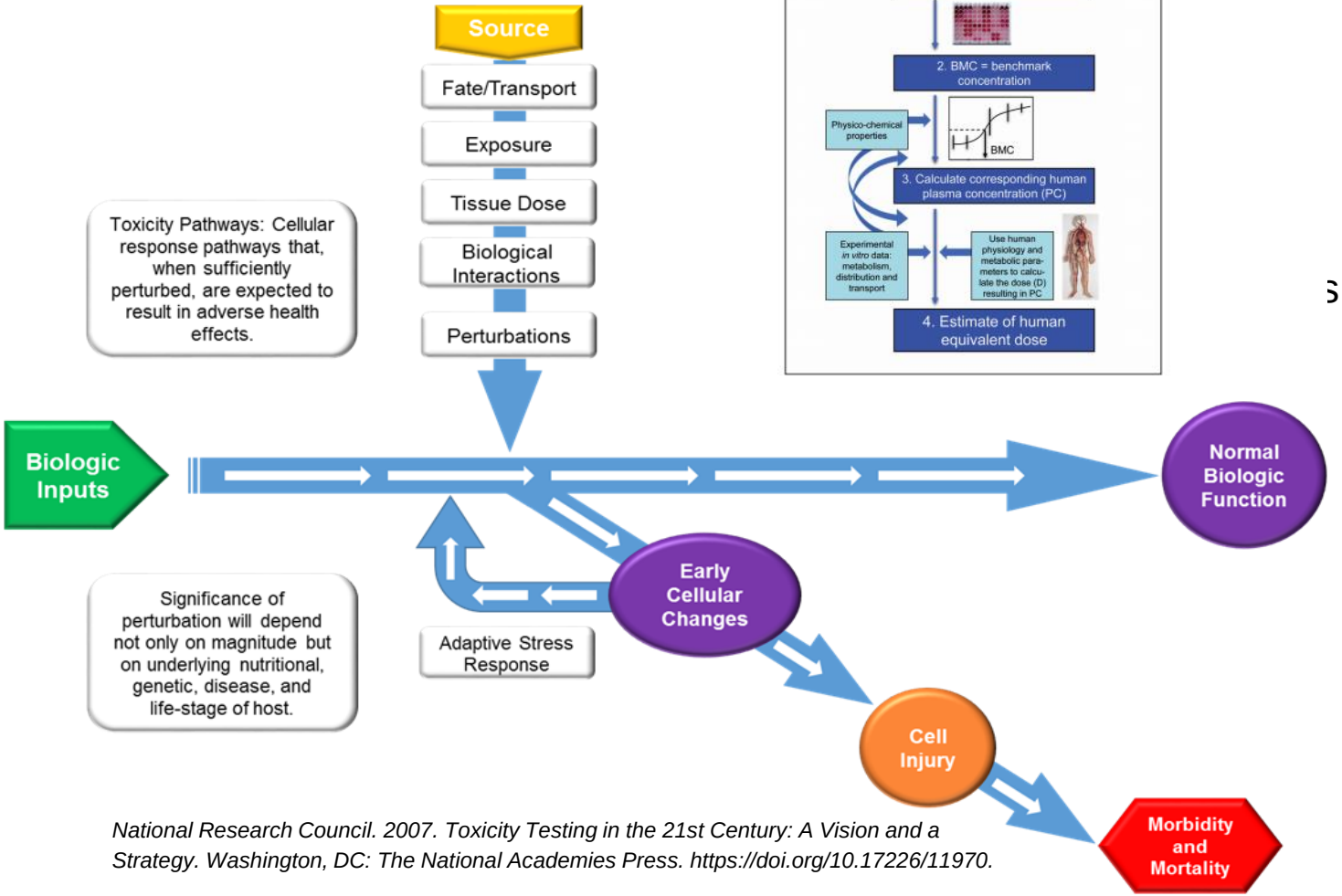
TT21C

IMPERIAL BRANDS

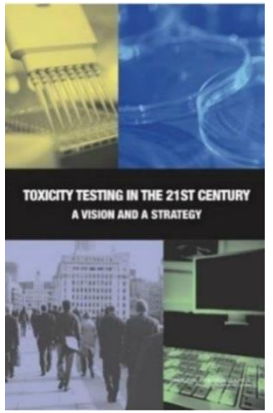
AGENDA

- Overview of Imperial Brands assessment frame work
- Assessment of NGPs in TT21C assays
  - BioMAP multiorgan biomarkers
  - Carcinogenicity (Bhas42 assay)
  - Regulatory assays
  - Reproductive assay
  - High Content Screening
  - CVD endpoints
  - COPD models
- Integration of mechanistic data in AOPs

WHAT IS A TT21C APPROACH



IMPERIAL BRANDS



National Research Council. 2007. Toxicity Testing in the 21st Century: A Vision and a Strategy. Washington, DC: The National Academies Press. https://doi.org/10.17226/11970.

OVERVIEW IMPERIAL BRANDS HARM REDUCTION FRAMEWORK FOR NGPS

2. BIOLOGICAL SCIENCE

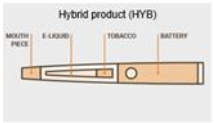
Once our Product Characterisation science has demonstrated that a product may have the potential to fit in the NGP portfolio, our laboratory-based in-vitro assays help determine if use of the product translates to reduced toxicity in human cells. Our approach is based on the US National Research Council's blueprint for Toxicity Testing in the 21st Century, or TT21C. We consider this approach more relevant, informative and ethical than previous assessment approaches used to evaluate toxicity. We do not test our products on animals.

In vitro assays including TT21C assays

NGP PLATFORMS TESTED AND SMOKING/VAPING REGIMES USED

Aim: To expose a range of TT21C assays to different NGPs smoke/aerosols trapped in PBS

Test products



Concentration µg/ml	3R4F	THP	HYP	myblu™	LOQ (µg/ml)
Nicotine	82.5	123.0	53.0	152.0	
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	0.5	<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	2.8	<LOQ	<LOQ	0.5

Exposure methodology :

3 x 10mls PBS impingers combined (30mls) per test

Collected using a VC10 (Vitrocell)

Concentrations: 3R4F – 1.8 puffs/mL NGP (x3) – 4 puffs/mL

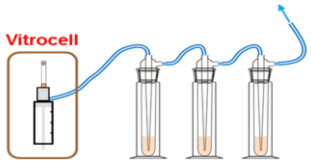
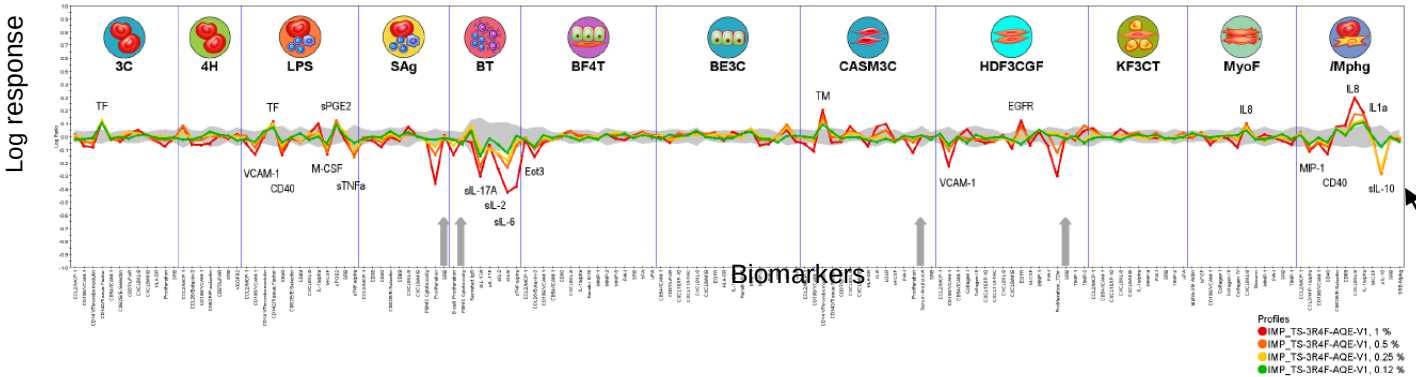


Figure 1
Bubbling exposure system

RESULTS: DISCOVERX ASSAY; SIGNIFICANT BIOMARKERS AFFECTED BY 3R4F

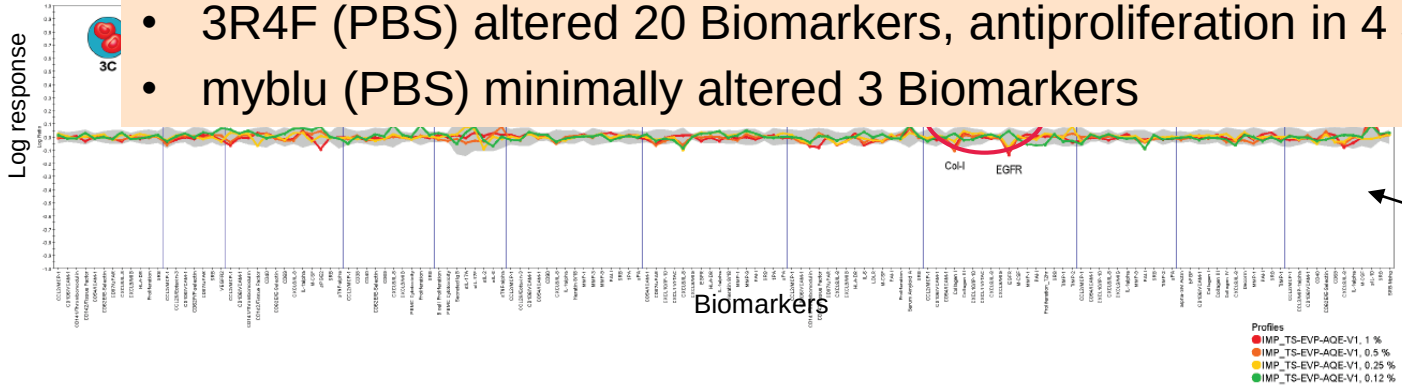
3R4F sample in PBS (0.012-1%)



Concentrations between 0.25-1% 3R4F-AQE- impacted inflammation:

Decreased Eotaxin 3, VCAM-1, sTNFα, MIP-1α;
increased IL-8, IL-1α, sPGE2),
tissue remodelling (**increased** EGFR),
haemostasis-activities
(**increased** TF, TM)

Myblu sample in PBS (0.012-1%)



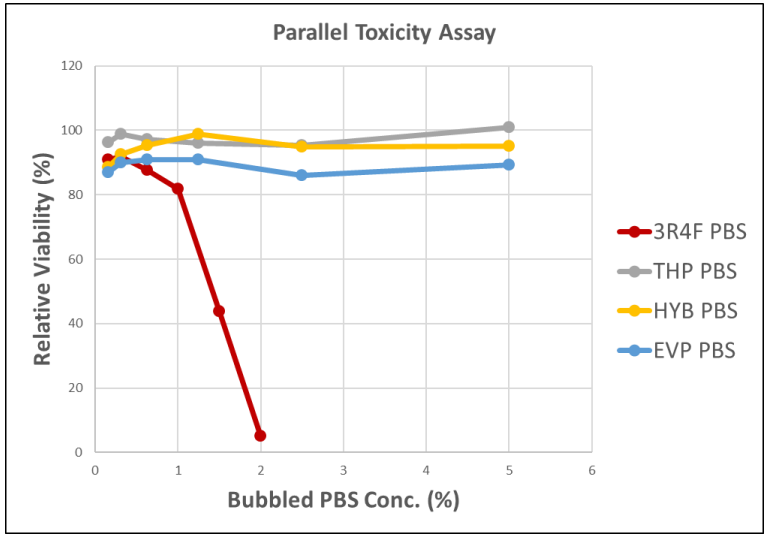
- 3R4F (PBS) altered 20 Biomarkers, antiproliferation in 4 systems
- myblu (PBS) minimally altered 3 Biomarkers

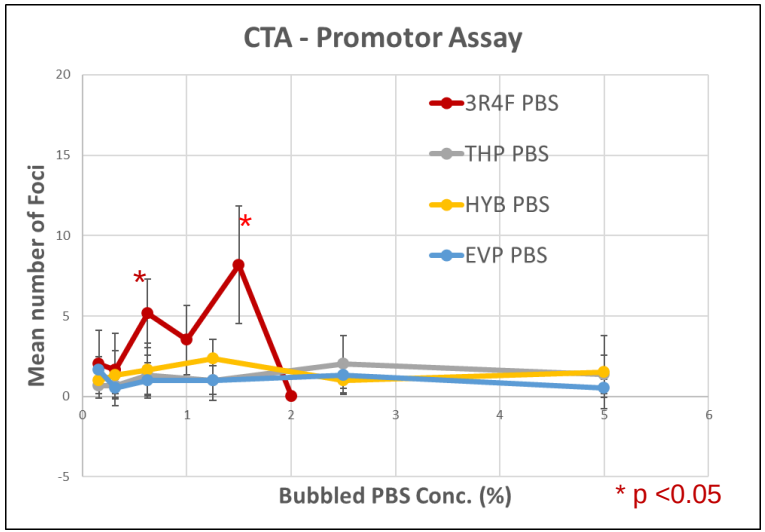
At 1% myblu had **very minor** isolated immunomodulatory activities (not seen across multiple cell systems). Not significant

BioMAP® Diversity PLUS®

Grey = control envelope, grey arrows = antiproliferation; * consistent biomarker changes are labelled with ID

RESULTS: BHAS-42 CELLULAR TRANSFORMATION ASSAY



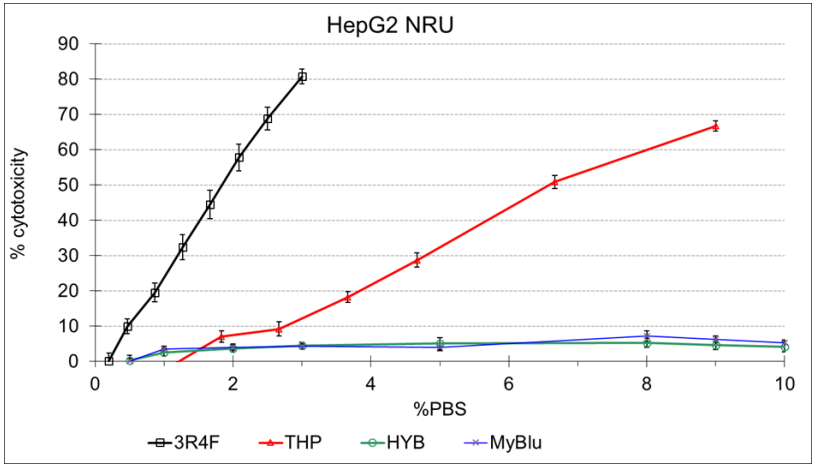


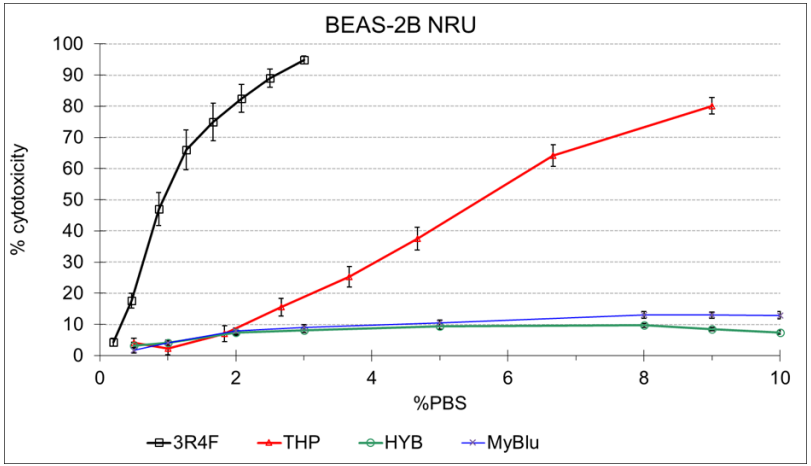
- 3R4F showed extensive cytotoxicity at PBS concentrations >1%
- Weak to no cytotoxicity observed for NGP PBS even at a max PBS concentration of 5%
- No promotor activity was observed for the PBS for all NGPs

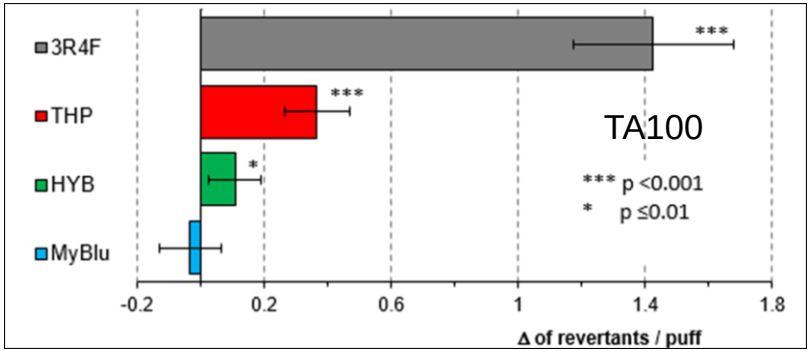
Mouse BALBc/3T3 derived cells

RESULTS: BUBBLED PBS IN NRU, AMES & IVMN

Bubbled PBS Biological response:

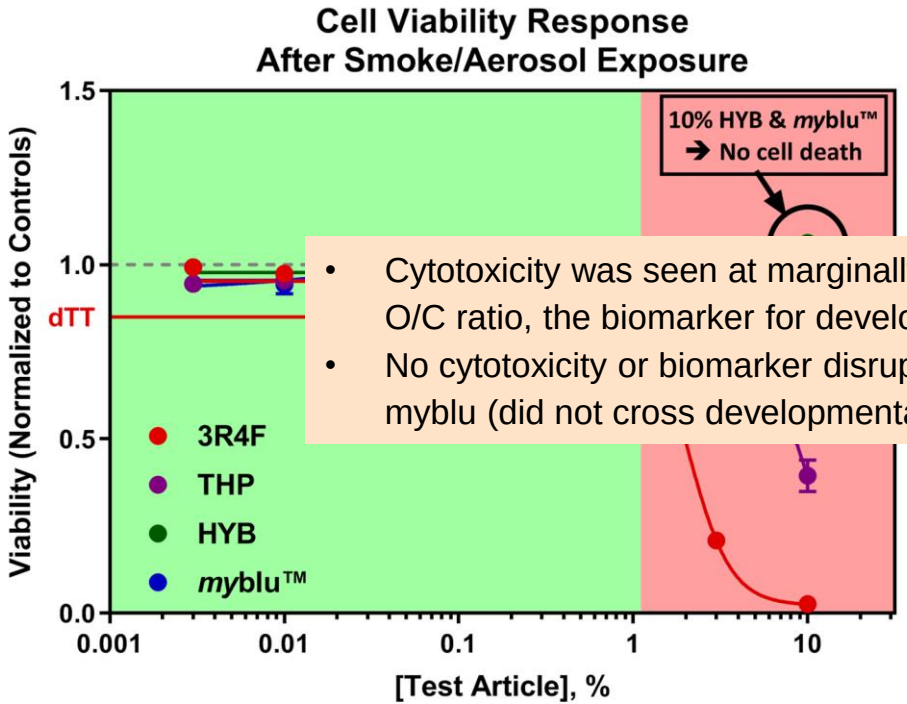


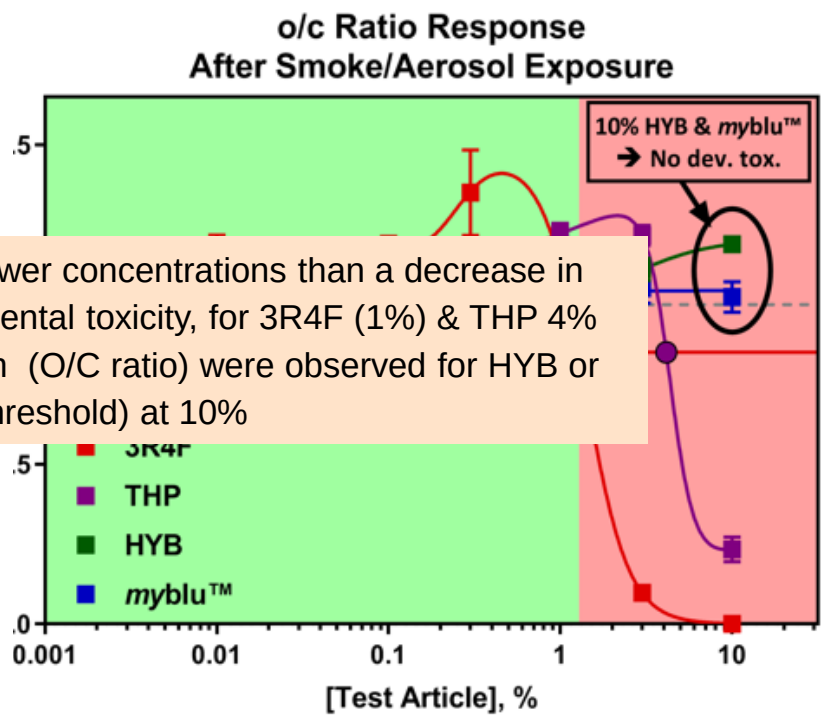




- Marked Cytotoxicity for 3R4F PBS (ET_{50}: 1-1.5%); less for THP (EC_{50}: 5.8-6.7%) and weak cytotoxicity for HYB and myblu (EC_{50}: >10%)
- Extracts were mutagenic in Ames TA100, except EVP extracts
- No extracts had any activity in IVMN assay (TK6 or V79)

RESULTS: DEVELOPMENTAL TOXICITY POTENTIAL





- Cytotoxicity was seen at marginally lower concentrations than a decrease in O/C ratio, the biomarker for developmental toxicity, for 3R4F (1%) & THP 4%
- No cytotoxicity or biomarker disruption (O/C ratio) were observed for HYB or myblu (did not cross developmental threshold) at 10%

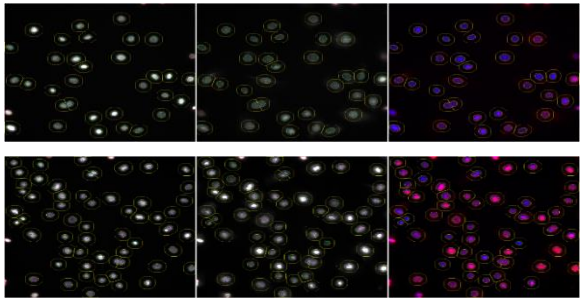
Human induced pluripotent stem (iPS) cells

RESULTS: HCS MORE ACTIVITY FOR 3R4F VS THP, NO ACTIVITY FOR HYB AND MYBLU

- Image based analysis on single cell level for marker quantification and / or structure analysis.
- Induction of different endpoints analysed and compared after treatment of NHBEs with bPBS from different NGP platforms and the reference cigarette 3R4F

Assay *	Endpoint No.	Biological endpoint	Cellular compartment	Output feature	Positive control
cytotoxicity	1	cell count	nucleus	valid cell count	-
Apoptosis	2	cytochrome C release	cytoplasm/ nucleus	ring average intensity	staurosporin
Gamma H2AX	3	Phosphorylation of histone-H2AX	nucleus	average circ intensity	etoposide
stress kinase	4	phospho-cJun	nucleus	average circ intensity	CCCp/stauro sporine for STTNFalpha for Long term
Inflammation	5	NFKB translocation	nucleus/ cytoplasm	Nucleus Cytoplasm Average Intensity Difference	TNFalpha
Thioltracker	6	GSH depletion/ oxidative stress	nucleus/ cytoplasm	Average circ. Intensity	H_2O_2

* All endpoints tested on 3 independent test days for each NGP platform and Reference Cigarette 3R4F



γH2AX staining

Significant HCS end points by time (4 and 24 hours)

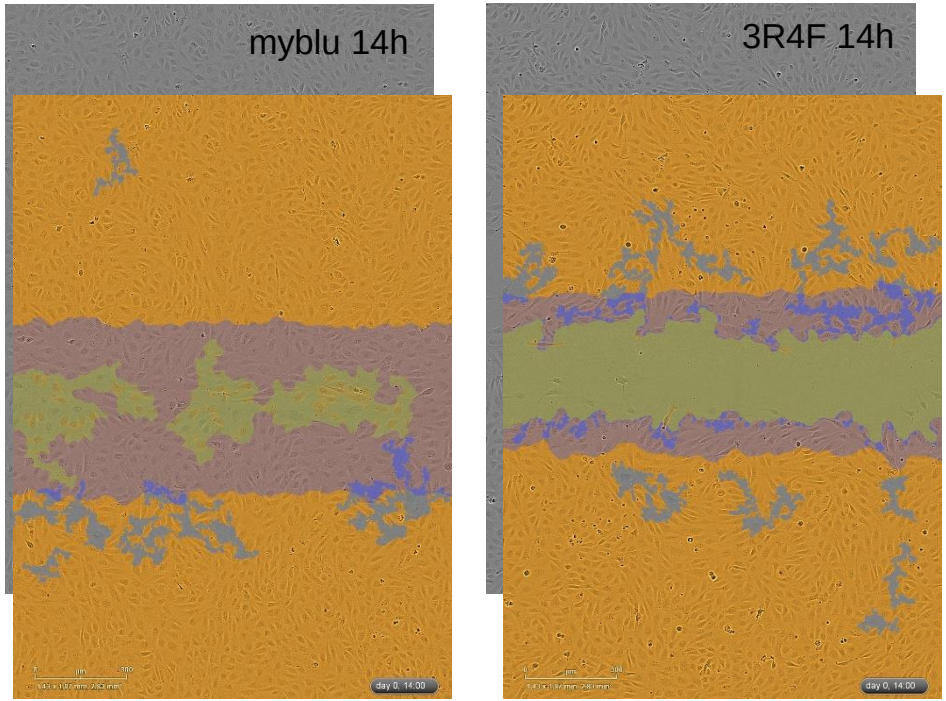
4 hour treatment	GH2AX	p-c-jun	NfKB	GSH (2h)
3R4F	3-4%	2.5%-4%	4%	2-5%
THP	-	6%	4.5-10%	-
HYB	-	-		-
myblu	-	-		-

24 hour treatment	Cytotox	GH2AX	p-c-jun	NfKB
3R4F	1-4%	3%-4%	4%	4%
THP	-	9%-10%	-	9% -10%
HYB	-	-	-	-
myblu	-	-	-	-

(see also poster by E. Trelles Sticken et al., and Presentation by Stefan Frentzel in this workshop)

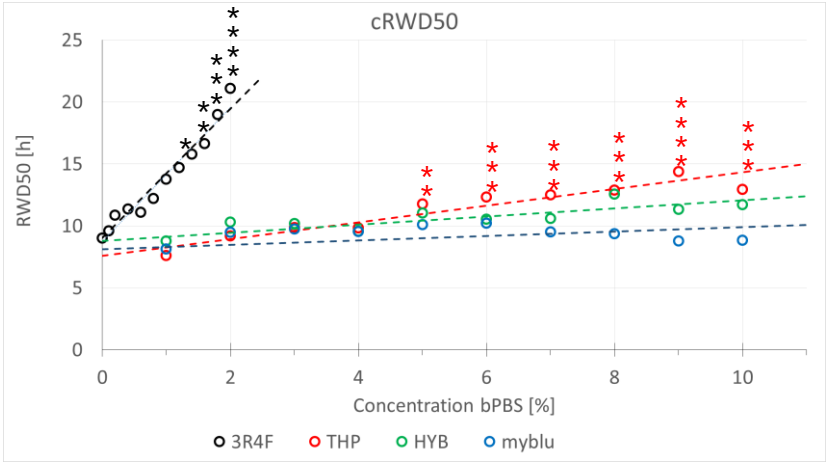
RESULTS: SCRATCH WOUND CLOSURE, SIGNIFICANT INHIBITION BY 3R4F AT 14 HOURS

CVD END POINTS (1)



HUVEC cells, initial scratch, confluence mask, cell free area due to migration

Concentration dependent relative wound density (RWD)



* p≤0.05 ** p≤ 0.01 ***p ≤ 0.005**** p ≤ 0.0001

RWD50= time needed to fill the scratch with cells up to 50% density.

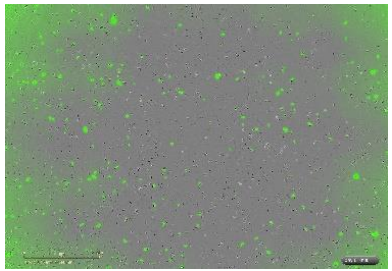
- 3R4F showed strong inhibition of healing activity while THP showed much lower but still significant inhibition.
- HYB and myblu bPBS did not influence the RWD in meaningful manner.

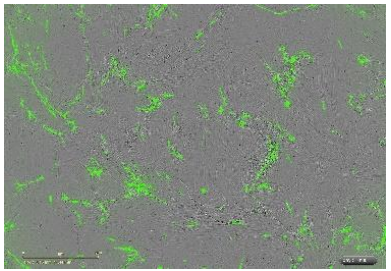
(see also poster by L Simms et al.)

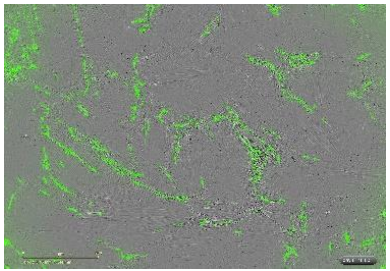
RESULTS: ANGIOGENESIS, SIGNIFICANT INHIBITION BY 3R4F

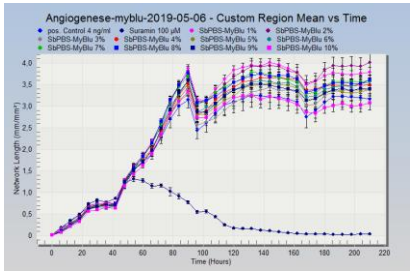
CVD END POINTS (2)

myblu 10% bPBS (no difference to negative control)



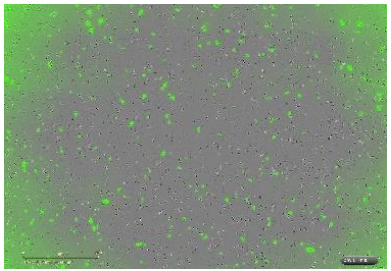


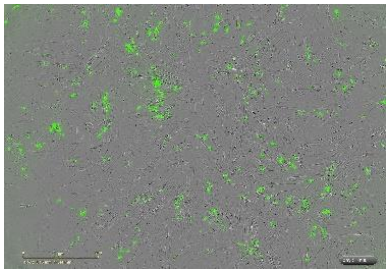


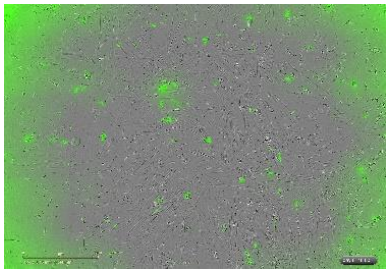


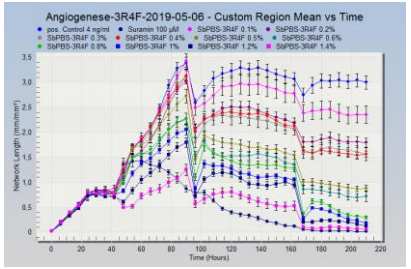
Day 0 Day 5 Day 8

3R4F 1.2% bPBS









Test system that mimics formation of new blood vessels.

- HUVECS stabile transformed with GFP marker seeded onto a feeder layer of human dermal Fibroblasts.
- Stimulation by VEGF
- Growth in presence of test substance over 8 days

RESULTS: 3D LUNG MODEL (4-WEEKS REPEATED EXPOSURE)

Significant effects for 3R4F 30, 60 & 90 puffs in 3D cultures

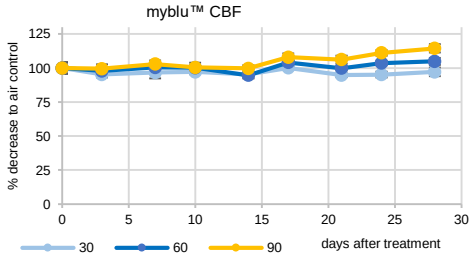
SAEIVS

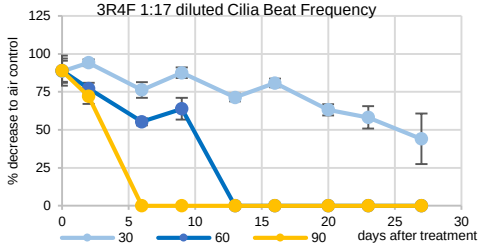
Smoke Aerosol Exposure *In Vitro* System (SAEIVS)

- Exposure of 3D cultures to 30, 60, 90 puffs from the different platforms
- Over 4 weeks every 2-3 days: EVP undiluted, 3R4F 1:17 diluted

Endpoints:

- Inflammatory panel as measured in a multiplex device (Markers IL-6, 8, 1β & TNF- α, MMP1, MMP3, MMP9)
- Ciliary beating frequency

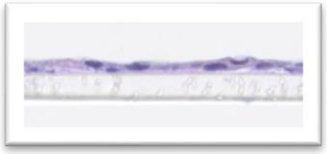




- Histology staining: H&E/ Alcian Blue

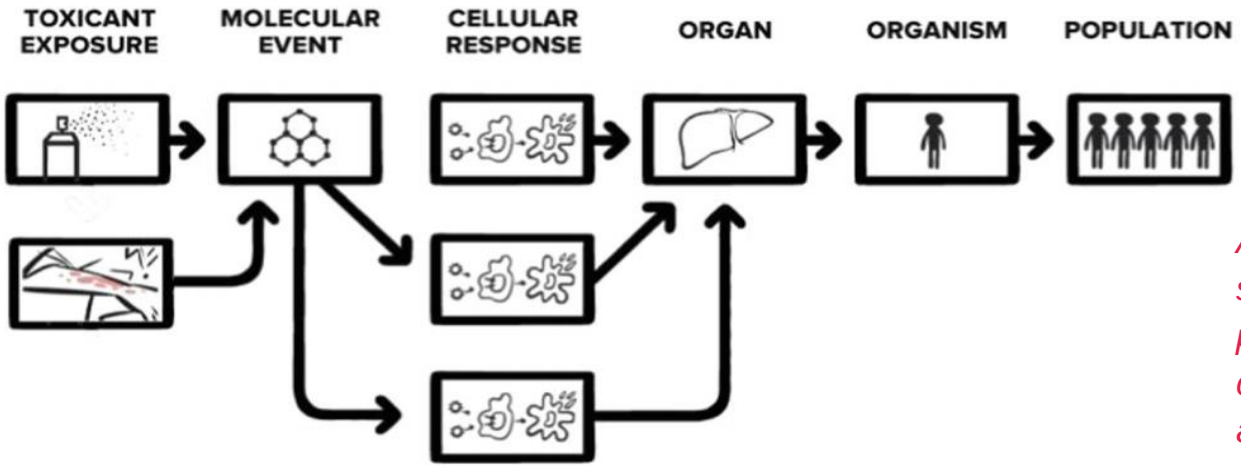
90Puffs myblu (4 weeks)

H&E/ Alcian Blue

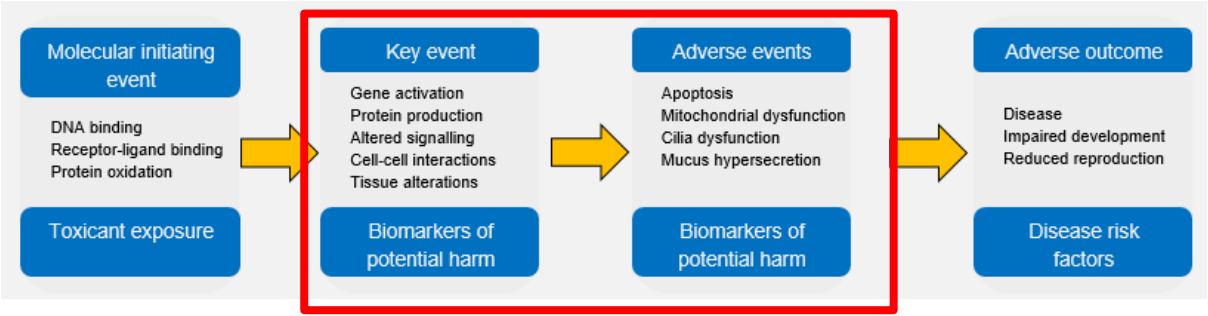


90Puffs 3R4F

USE OF AN AOP TO ORGANISE TT21C MECHANISTIC DATA



AOPs are deliberate simplifications of biological pathways, to enable communication/discussion around complex processes



AOP = Adverse Outcome Pathway

WHERE DO CURRENT TT21C ASSAYS FIT IN TO AN AOP?

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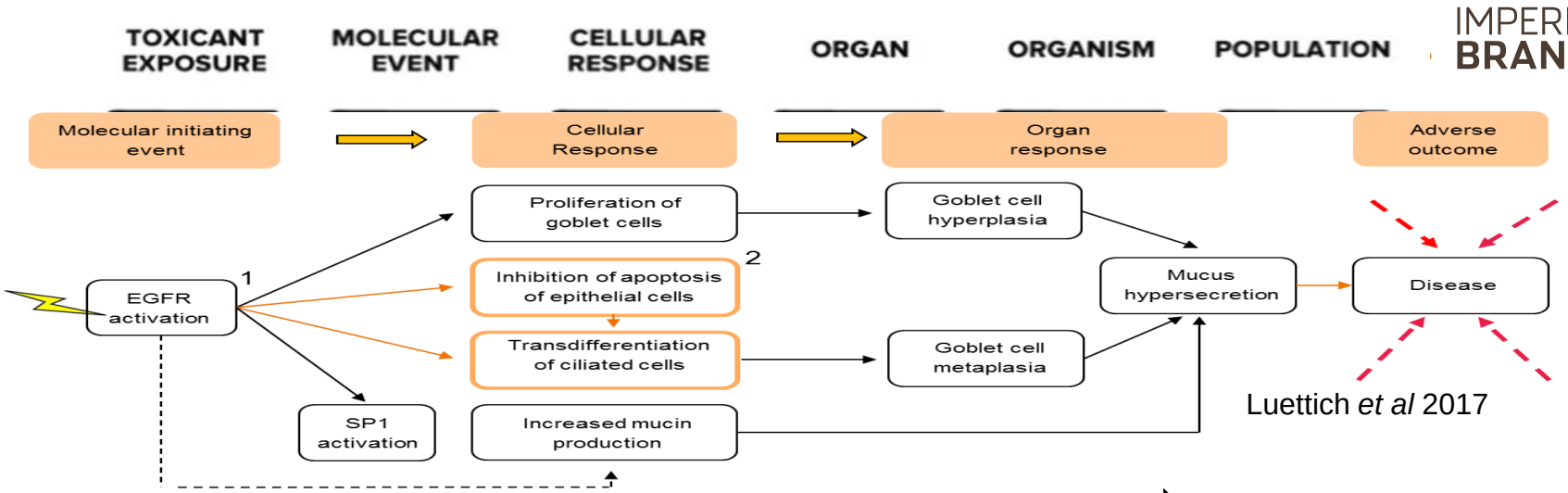
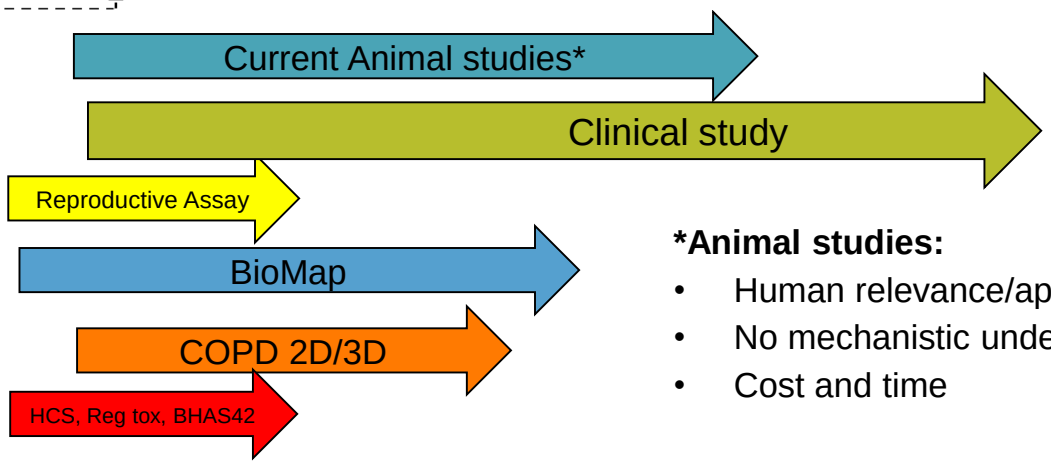


Luettich *et al* 2017

Regardless of the AOP there are multiple key events and interactions across many pathways that can lead to the same measured outcome.



***Animal studies:**

- Human relevance/applicability?
- No mechanistic understanding
- Cost and time

CONCLUSIONS

- Results we have obtained show that the TT21C Assays are sensitive to smoke and a range of aerosols
- Use of TT21C relevant assays allows for a mechanistic based understanding of potential toxicity of NGPS
- Challenges include further integrating these assays, creating AOPs
- These TT21C assays allow an overall ranking of products in terms of potential harm, based on a weight of evidence approach

ACKNOWLEDGEMENTS

Thanks to colleagues in Imperial Brands Science Team, Laboratory Network, and CROs for their contributions to this presentation