The use of human cells for ‘R’ end point for EUTPD2 data requirements from a TT21C perspective

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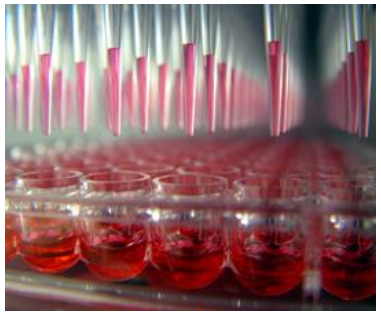
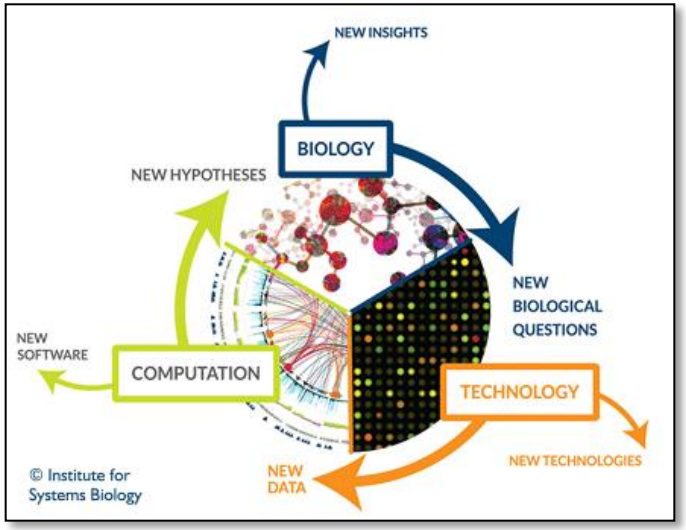
Land mark paper NRC (2007) led to a significant paradigm shift in toxicology

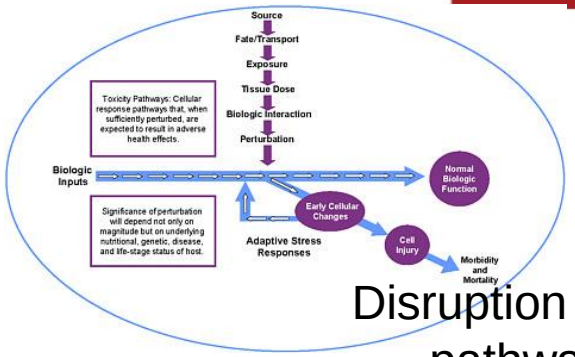
Toxicity testing in 21st Century: A vision and a strategy



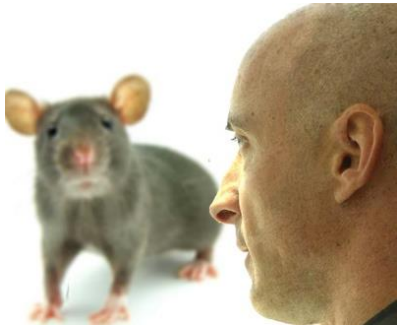
High Throughput testing



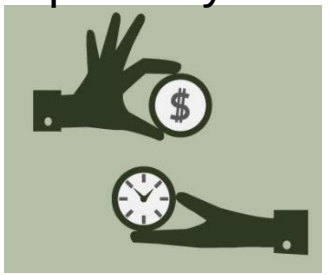
Systems biology, computational and omics approaches



Disruption of key pathways



Human relevance



Significantly reduced costs and time

Opinion 1: 15 priority ingredients identified

ANNEX

to the

Commission Implementing Decision

laying down a priority list of additives contained in cigarettes and roll-your-own tobacco subject to enhanced reporting obligations

Priority list of additives used in cigarettes and roll-your-own tobacco subject to enhanced reporting obligations

Additive	Chemical formula (if applicable)	CAS number(s) applicable to the substance (not exhaustive)
Carob bean		9000-40-2, 84961-45-5
Cocoa		84649-99-0, 84649-99-3, 95009-22-6, 8002-31-1
Diacetyl	$C_4H_6O_2$	431-03-8
Fenugreek		68990-15-8, 977018-53-3, 84625-40-1
Fig		90028-74-3
Geraniol	$C_{10}H_{18}O$	106-24-1, 8000-46-2
Glycerol	$C_3H_8O_3$	56-81-5
Guaiacol	$C_6H_4(OH)(OCH_3)$	90-05-1
Guar gum		9000-30-0
Liquorice		68916-91-6
Maltol	$C_6H_6O_3$	118-71-8
Menthol	$C_{10}H_{20}O$	2216-51-5, 15356-60-2, 89-78-1, 1490-04-6, 8006-90-4, 68606-97-3, 84696-51-5, 8008-79-5
Propylene glycol	$C_3H_8O_2$	57-55-6
Sorbitol	$C_6H_{14}O_6$	50-70-4
Titanium dioxide	TiO_2	13463-67-7, 1317-70-0,

Opinion 2 Assays for the end point released July 2016 (SCHEER):

- CMR properties for both neat additives and additive when pyrolysed, to a **significant and measurable degree**
- Focus on *in silico*, *in vitro* with *in vivo* under limited circumstances.
- Applies to FMC and RYO products only
- Reproductive assays mentioned once under oestrogenic activity

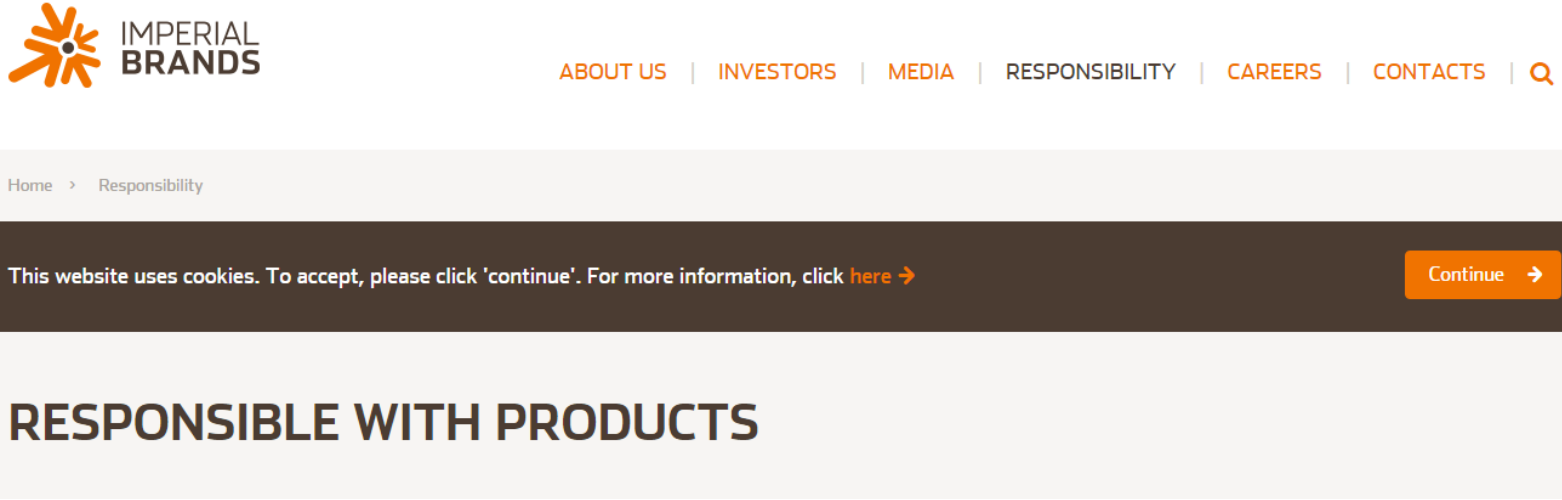
CMR – Carcinogenic, Mutagenic and Reproductive
SCHEER – Scientific Committee Health and Environmental and Emerging Risks

Rodents are not a good model for development effects

- Public health bodies (US Surgeon General 2010) and EUTPD/2001 have stated the adverse reproductive effects of smoking in humans
- Limited number of *in vivo* inhalation studies for cigarettes
- No reproductive effects of reference cigarettes*
  - Sole observation; delayed ossification associated reduced body weights observed
- Current Research is focussed on oxidative stress and utilisation of TT21C technologies

*Carmines *et al*., 2003, 2008; Gaworski *et al*., 2004, TT21C – Toxicity Testing in the 21st Century

ITL does not test on animals



Current *in vivo* / *ex vivo* developmental screening assays

Reproductive Toxicity Study types:

- Segment 1: Fertility and general reproductive performance
- Segment 2: Teratogenicity
- Segment 3: Peri and postnatal development

ECVAM validated assays

- Mouse Embryonic stem cell test mEST
- Rodent whole embryo culture
- Micromass culture

Alternative assay
(working towards regulatory acceptance)

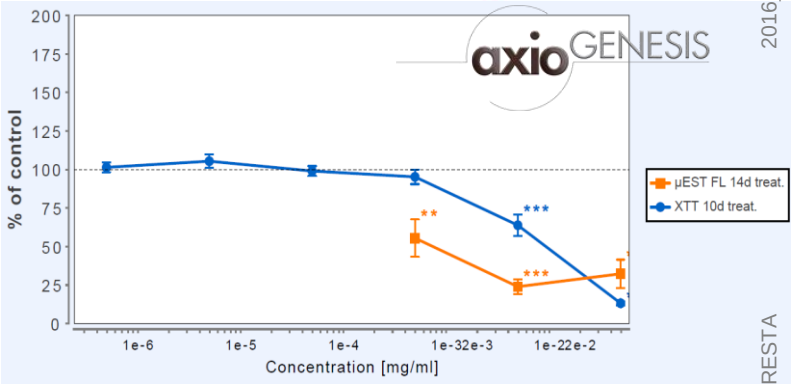
- Metabolomics approach using human cells

Stemina BIOMARKER DISCOVERY

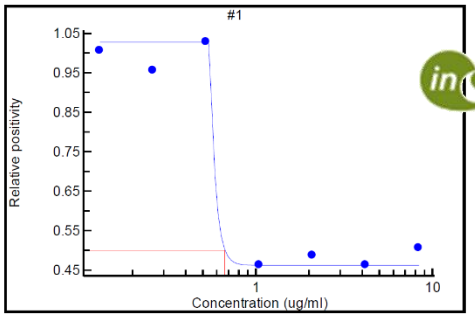
ECVAM: European Centre for the Validation of Alternative Methods

TPM in mouse embryonic stem cell assays (EST) gave a positive indication for embryotoxicity

- Preliminary investigation with TPM in mouse embryonic stem cell assays (EST)
  - Axiogenesis (µEST) -> embryotoxic
  - InSphero (EasyEST) -> weak embryotoxicity
- Results from both assays were comparable
- Note: this is a mouse stem cell line
  - In keeping with TT21C we also investigated a human derived alternative



ID50 – ESD3



inSphero
Biomimetic Microtissue Technology

ID50, 50% inhibition of cell differentiation; ESD3 mouse embryonic stem cell line

Alternative assay utilising Human induced pluripotent stem (iPS) cells

- Assay can be used to measure metabolic disruption
  - Which may lead to developmental toxicity
- Specifically it measures the ratio of two amino acids, ornithine and cysteine (o/c)
  - Metabolomics studies have indicated that known developmental toxicants are correlated with decreases in these two amino acids*
  - Decreases below 0.85 (fold changes) indicates metabolic imbalance linked to cellular stress
- Application for validation by ICCVAM being submitted.
  - Assay will be considered as part of Horizon 2020 project, which has a focus on reproductive end points (EU Toxrisk).

*Egnash *et al*. A biomarker-based developmental toxicity screen using human induced pluripotent stem cells for compound prioritization. Poster presented at: Society of Toxicology (SOT) Annual Meeting 2014.

High accuracy of iPSC for o/c ratio for known human reproductive toxicants

- Internal evaluation of a diverse set of 80 chemicals
  - 45 Developmental Toxicants,
  - 35 Non-Developmental Toxicants
- Compound set included:
  - Pharmaceuticals, Agrochemicals, Cosmetics, Industrial and Environmental Chemicals

Internal Validation Performance

Accuracy	Sensitivity	Specificity
85%	81%	89%

- Assay has been used by the US EPA to screen the ToxCast chemical library
- 1066 chemicals screened

Preliminary ToxCast Performance

Accuracy	Sensitivity	Specificity
82%	71%	100%

EPA Environmental Protection Agency; ToxCast EPA screening initiative to prioritise chemicals

devTOX^{qP} is highly predictive across birth defect lineages:

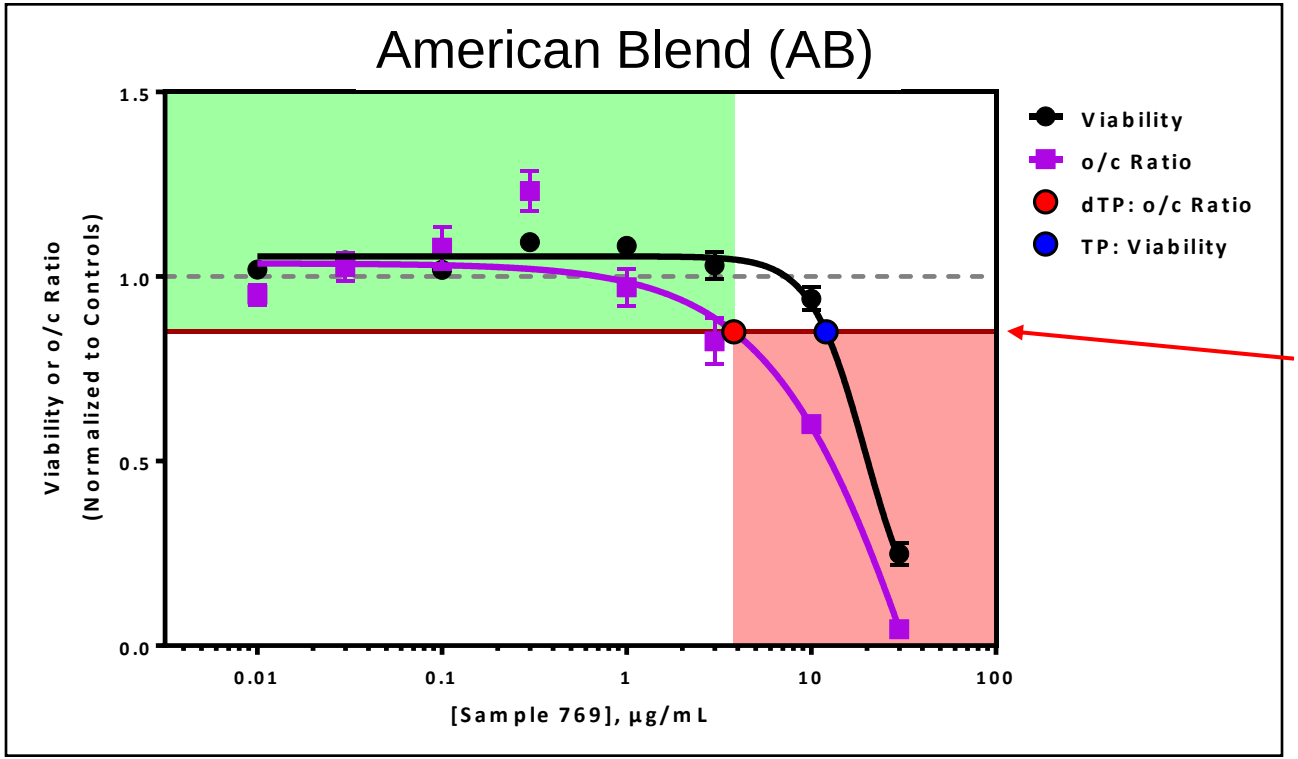
Birth Defect Category	# of Compounds Evaluated	Accuracy
Cardiovascular	12	83%
Central Nervous System	23	83%
Craniofacial	26	88%
Limb	15	93%
Skeletal	32	78%
Urogenital	8	88%
Other (e.g., Eye, Gastrointestinal)	4	75%
Embryo/Fetal Death	15	87%
Growth Restriction	19	74%
Skeletal Variations	7	100%
Endocrine Disruptors	**20**	**75%**

Investigation of TPM +/- additives with DevTox^{qp} assay

- Does the assay work with TPM?
- Is it sensitive enough to detect differences between different products (+/- additives)?

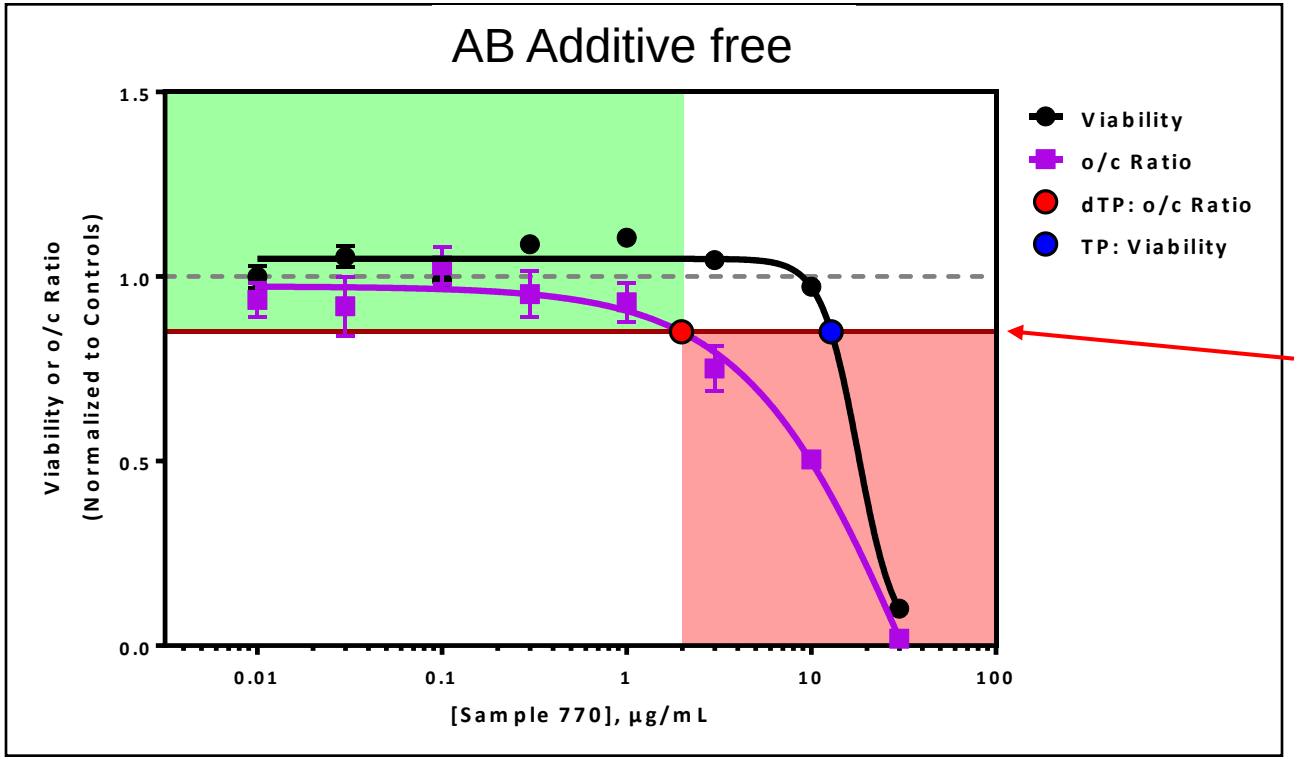
We analysed TPM from two different commercial cigarettes (+/- additives)

devTOX^{qP} Results: Full flavour AB



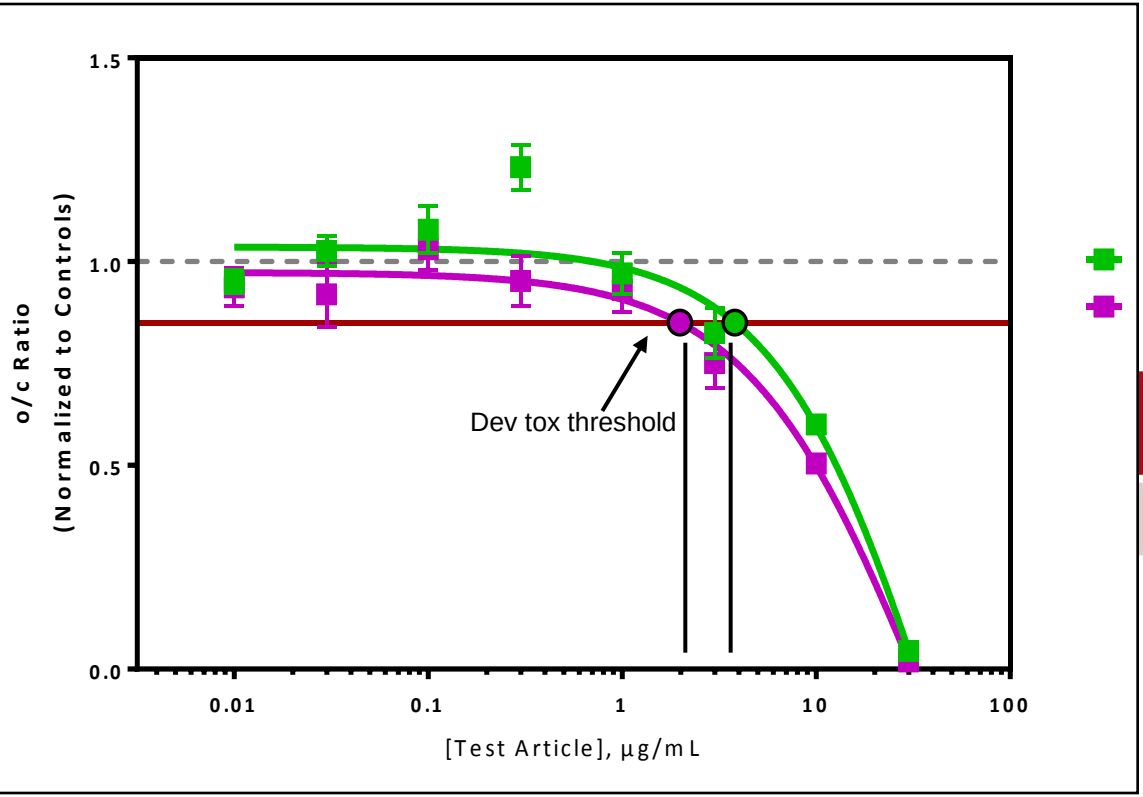
0.85 = Developmental toxicity threshold

devTOX^{qP} Results: AB Additive free



0.85 = Developmental toxicity threshold

Preliminary results indicate that there were no effects of additives on developmental toxicity



Sample (TPM)	Dev Tox quick predict (µg/ml)
AB (Additives)	3.8
AB (Add Free)	2.0

p value Hill slope	p value IC_{50}
0.9891	0.9414

Comparison of the two TPMs:

- No significant differences in Developmental Toxicity potential curves (O/C ratio).

IC_{50}, calculated 50% reduction in cell viability

Summary

- The use of animals for the R end point is unnecessary as they do not accurately predict findings in humans*
- The Mouse Embryonic Stem cell test is the only validated *in vitro* teratogenicity assay that does not utilise *ex-vivo* tissues
- The IPSC Stemina assay is preferable to the mouse EST:
  - Due to direct relevance to humans (TT21C)
  - Lack of interspecies differences and therefore easier extrapolation
  - It also has the ability to detect a wide range of developmental endpoints
  - Preliminary work indicates that this method is suitable for work with TPM

* Bailey et al., (2005) The future of teratology research is *in vitro*. Biog Amines 19 (2): 97-145

Acknowledgements

- Jessica Palmer, Principle Scientist, Stemina.com www.**stemina**.com (JPalmer@stemina.com)

- EST assays

devTOX^{qP} compared to Other Model Systems

Model System	Number of Compounds	devTOX^{qP} Concordance with Model	Accuracy**	
			Model	devTOX^{qP}
Rodent*	35	0.74	0.86	0.89
Rabbit*	28	0.79	0.79	0.86
Mouse EST	23	0.65	0.74	0.91
Zebrafish	24	0.75	0.75	0.92
Whole Embryo Culture	26	0.69	0.73	0.96

All values based on literature review of other model systems and known human developmental toxicants

*Current gold standard required model **Assay accuracy compared to human developmental toxicant