Safety Assessment without Animal Testing: Progress in Industry

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Unilever

Safety Assessment without Animal Testing

- Background
- The Past
- The Present
- The Future

Ensuring Safe Ingredients for Foods, Drinks and Cosmetic Products

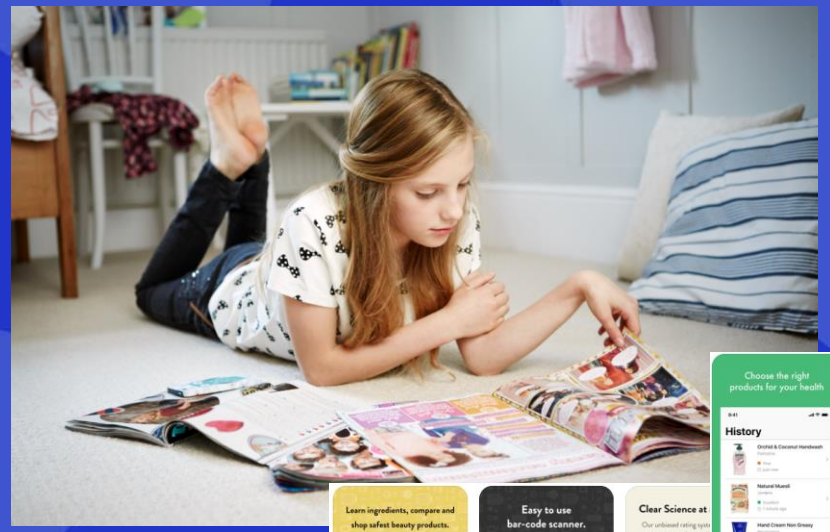
Risk Based Approach:
Considers both the hazard and the exposure to evaluate the risk

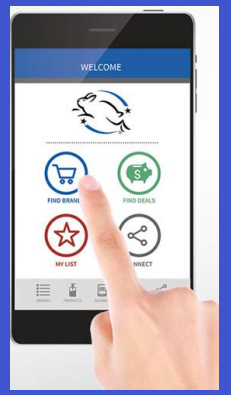
Can we safely use *x* % of ingredient in product or *x* t per annum?

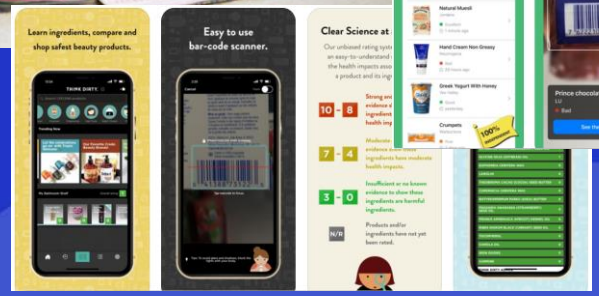
For **consumers**; **workers**;
the **environment**

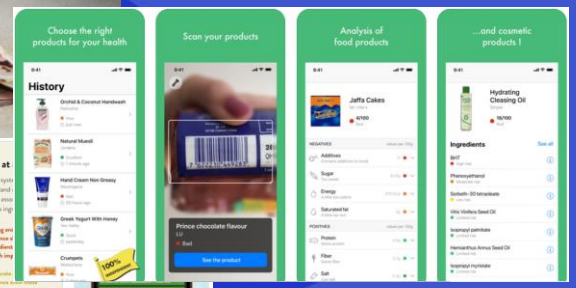
Toxicology has been undergoing a revolution

All Consumers Want Safe Products But Many Want Them Not to be Tested on Animals + Transparency









CERTIFIED VEGAN
VEGAN.ORG

NO CRUEL COSMETICS

NOT TESTED ON ANIMALS

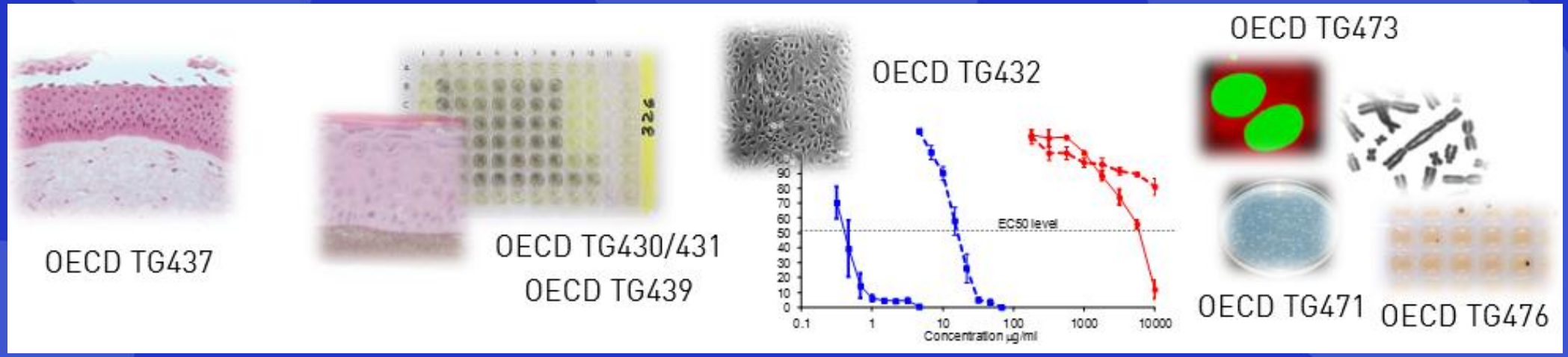
PETA
Cruelty-Free

Shop Cruelty-Free
www.LeapingBunny.org

PETA
Cruelty-Free and Vegan

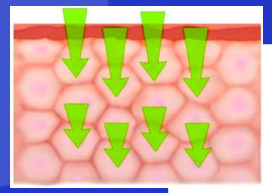
EUROPEAN VEGETARIAN UNION
V-LABEL.EU
VEGAN

Use of Existing OECD *In Vitro* Approaches

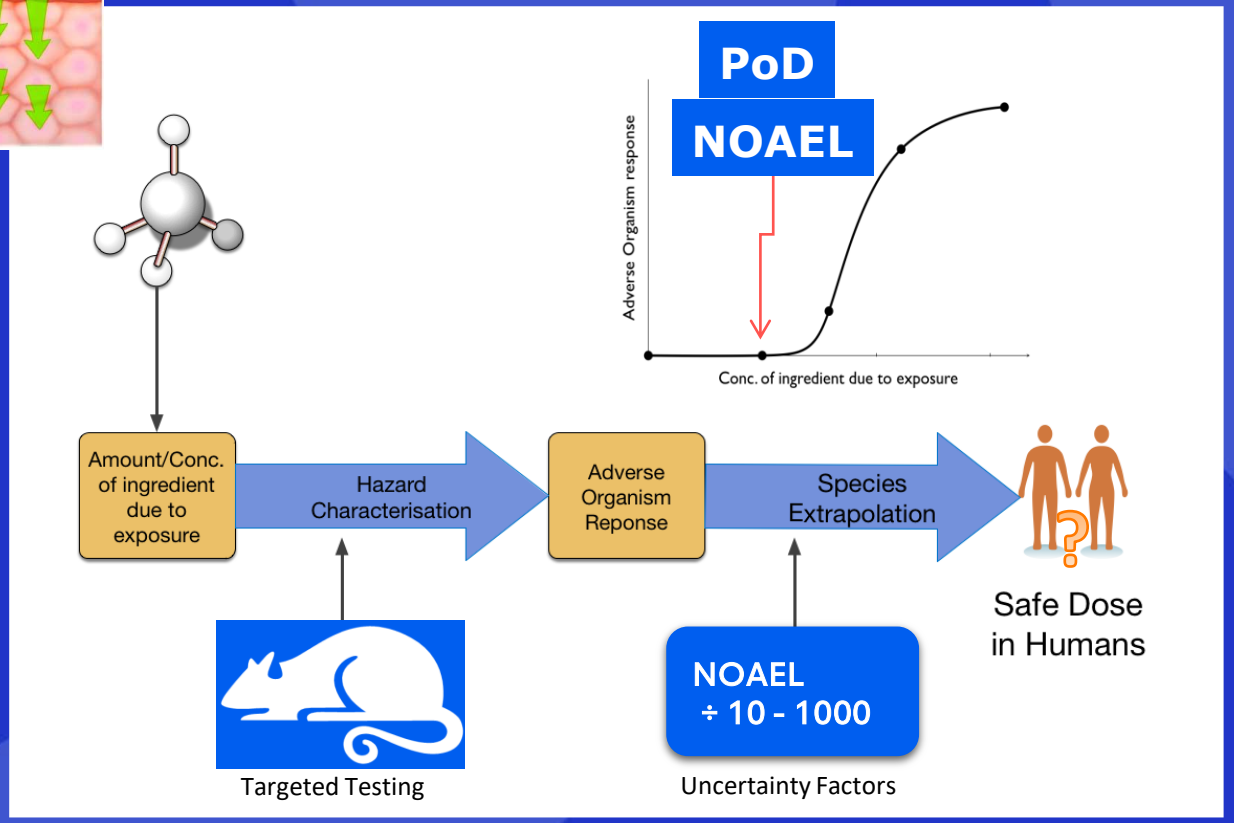


Skin and eye irritation; skin sensitization; phototoxicity; mutagenicity

What About Systemic Toxicity?



Is it safe?

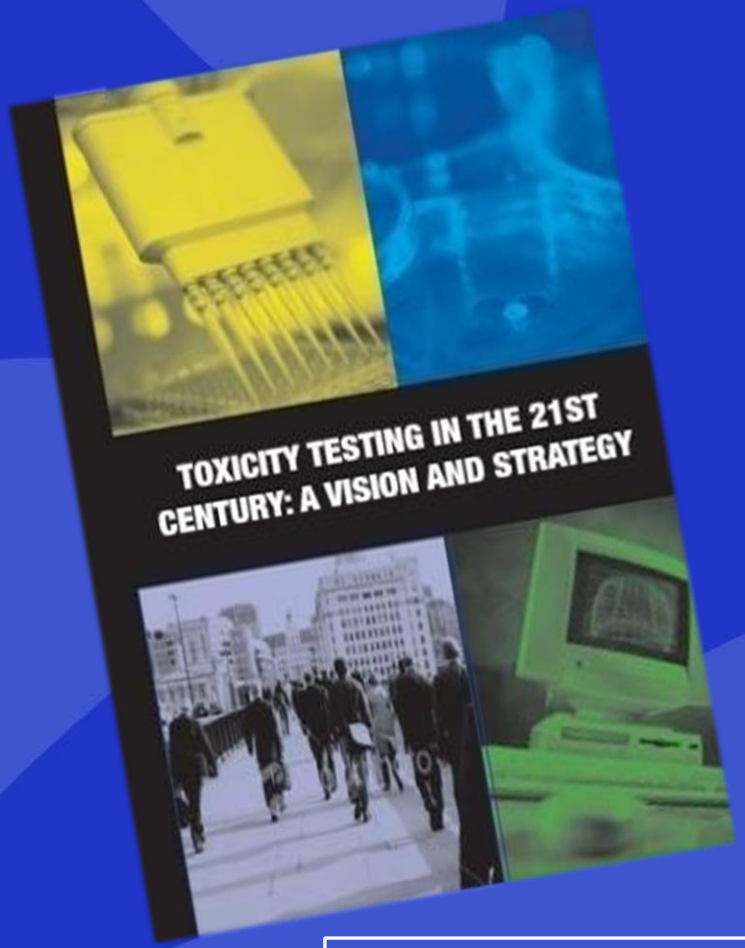


e.g. 90 Day Repeat Dose Study

A new non-animal paradigm is needed...

...but replacement of animal test data is not the answer

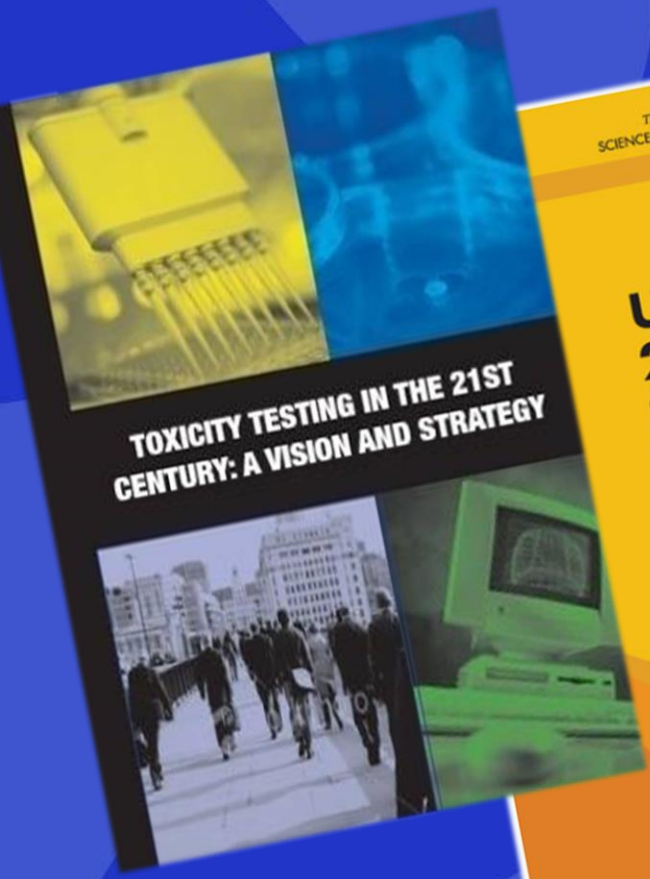
2007 Toxicity Testing in the 21st Century (TT21C)

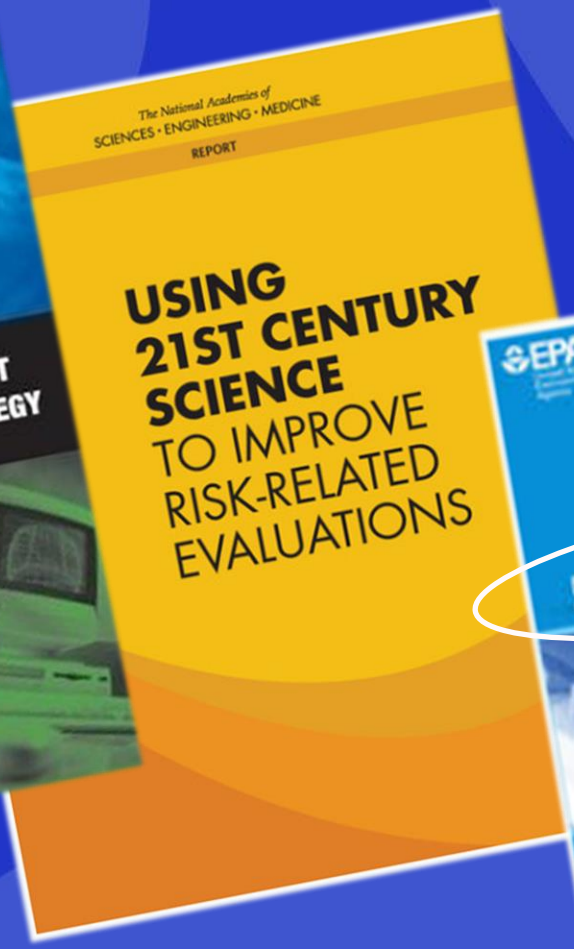


"Advances in toxicogenomics, bioinformatics, systems biology, and computational toxicology could transform toxicity testing from a system based on whole-animal testing to one founded primarily on in vitro methods that evaluate changes in biologic processes using cells, cell lines, or cellular components, preferably of human origin."

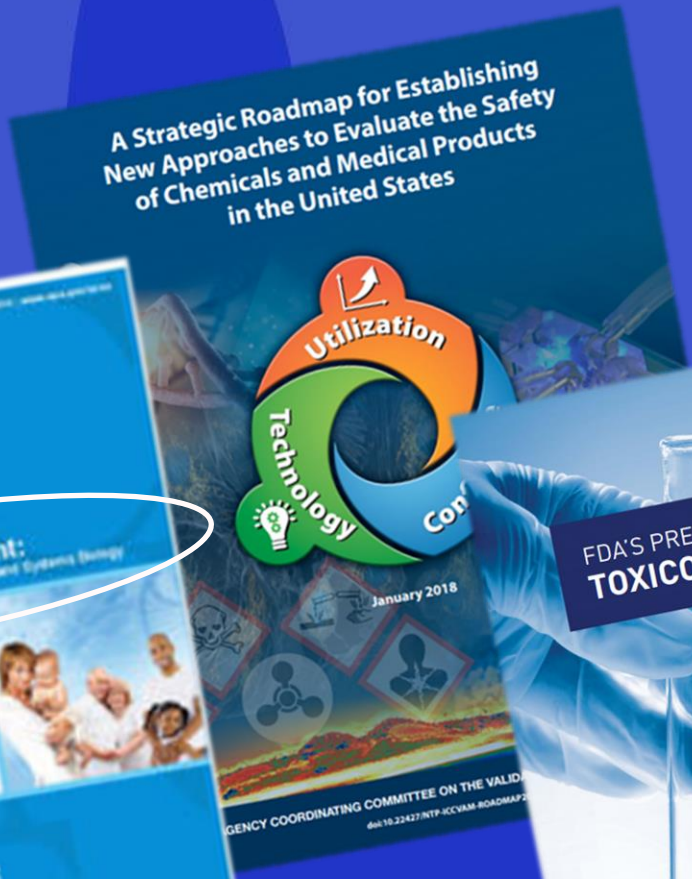
Perturbation of 'toxicity pathways' and stress responses

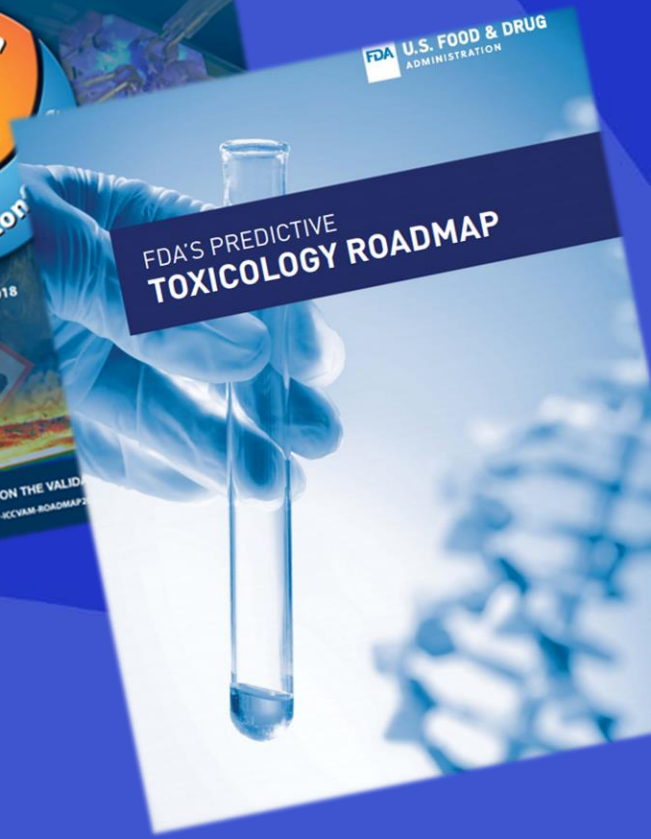
TT21C + NGRA


TOXICITY TESTING IN THE 21ST CENTURY: A VISION AND STRATEGY


The National Academies of
SCIENCES • ENGINEERING • MEDICINE
REPORT
USING 21ST CENTURY SCIENCE TO IMPROVE RISK-RELATED EVALUATIONS


EPA
Next Generation Risk Assessment:
Final Report


A Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States
Utilization
Technology
January 2018


FDA U.S. FOOD & DRUG ADMINISTRATION
FDA'S PREDICTIVE
TOXICOLOGY ROADMAP

Principles of NGRA from ICCR

4 Main overriding principles:

- The overall goal is a human safety risk assessment
- The assessment is exposure led
- The assessment is hypothesis driven
- The assessment is designed to prevent harm

3 Principles describe how a NGRA should be conducted:

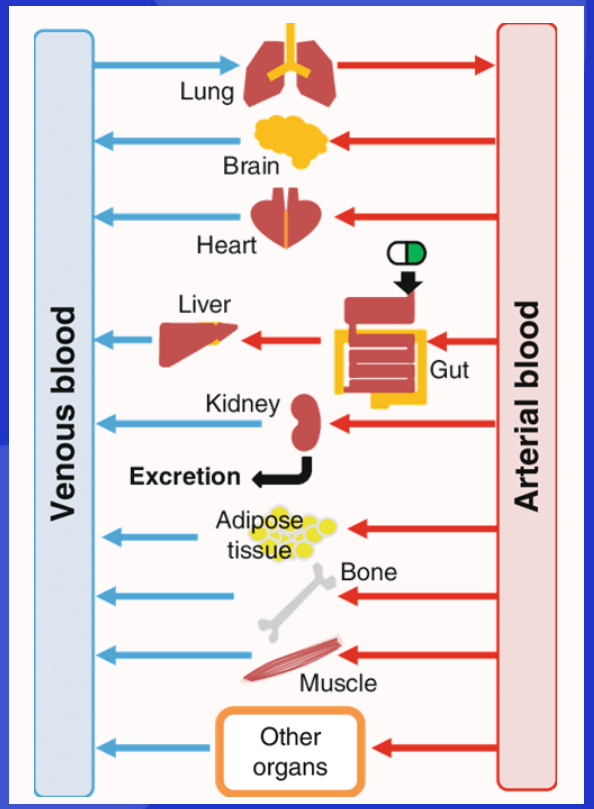
- Following an appropriate appraisal of existing information
- Using a tiered and iterative approach
- Using robust and relevant methods and strategies

2 Principles for documenting NGRA:

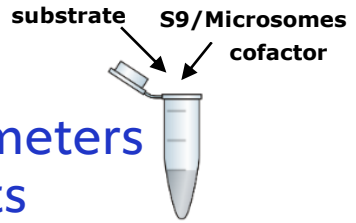
- Sources of uncertainty should be characterized and documented
- The logic of the approach should be transparently and documented

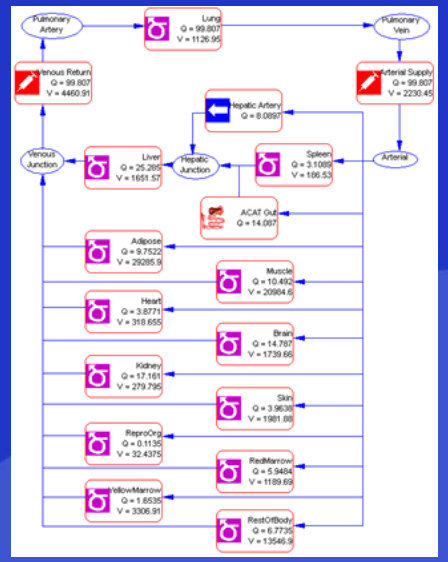
Dent *et al* ., (2018) *Comp Tox* 7:20-26

PBK (Physiologically Based Kinetic) Modelling



Model Input:
Physiological parameters
Partition coefficients
Kinetic constants (in vitro)



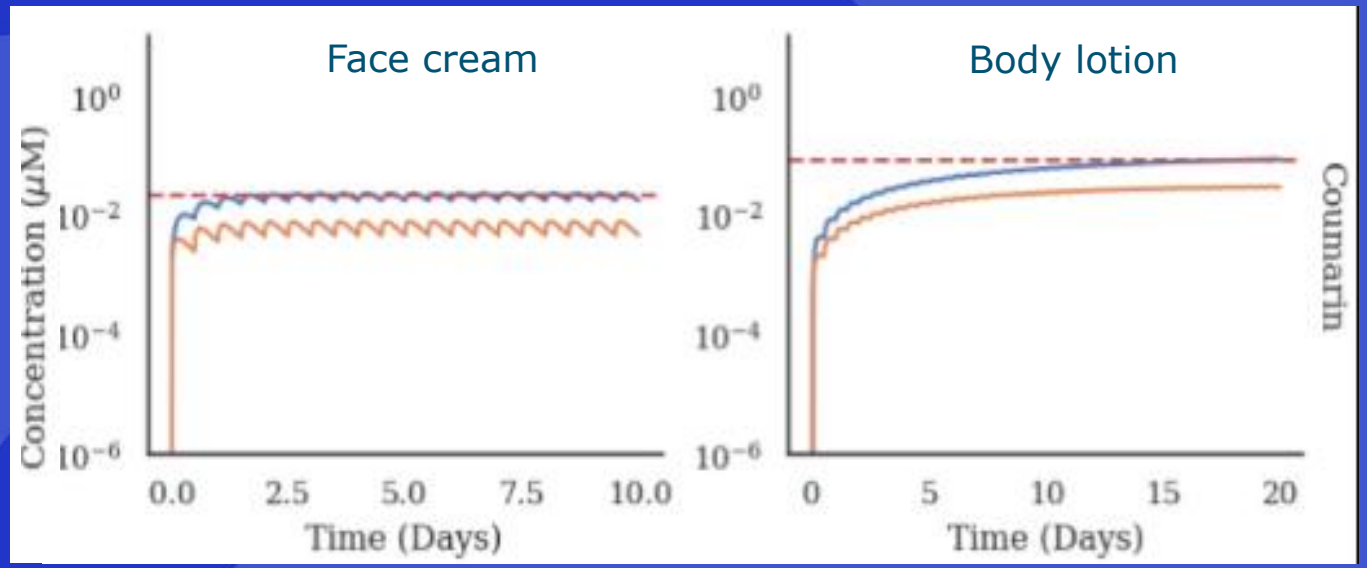


Uptake

Transport from arterial to venous blood

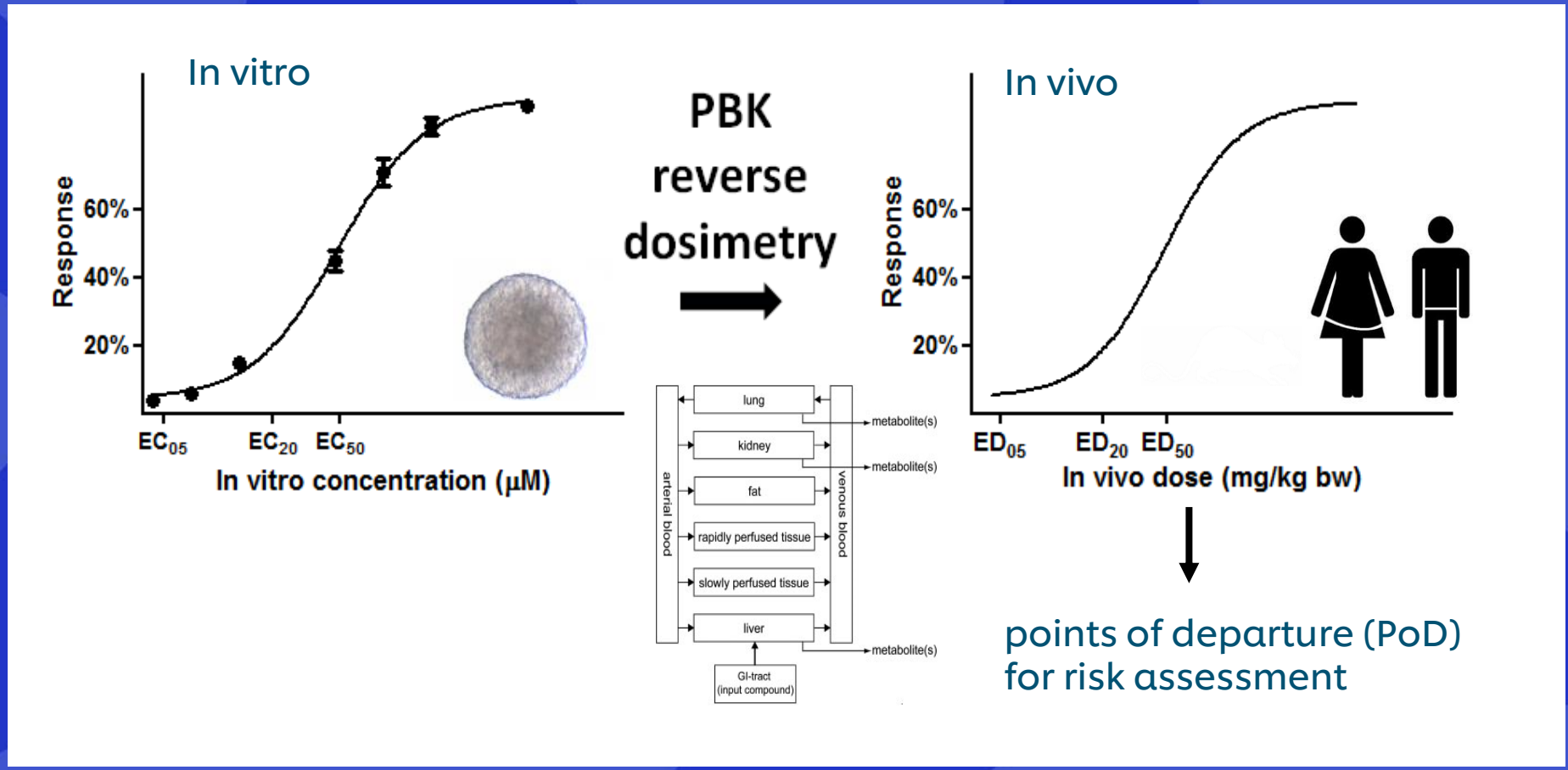
Metabolism

$$dA/dt = + K_A * A_{GI} + QL * (CA - CV) - V_{max} * CL / (K_m + CL)$$



Moxon *et al.*, (2020) *TIV* 63

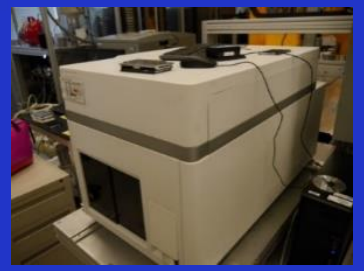
One Interpretation of TT21C: Quantitative *in vitro* to *in vivo* extrapolation



Another Interpretation: Tox21/ToxCast ~700 HTS Biological Pathways Assays






https://www.epa.gov/chemical-research/toxicity-forecasting

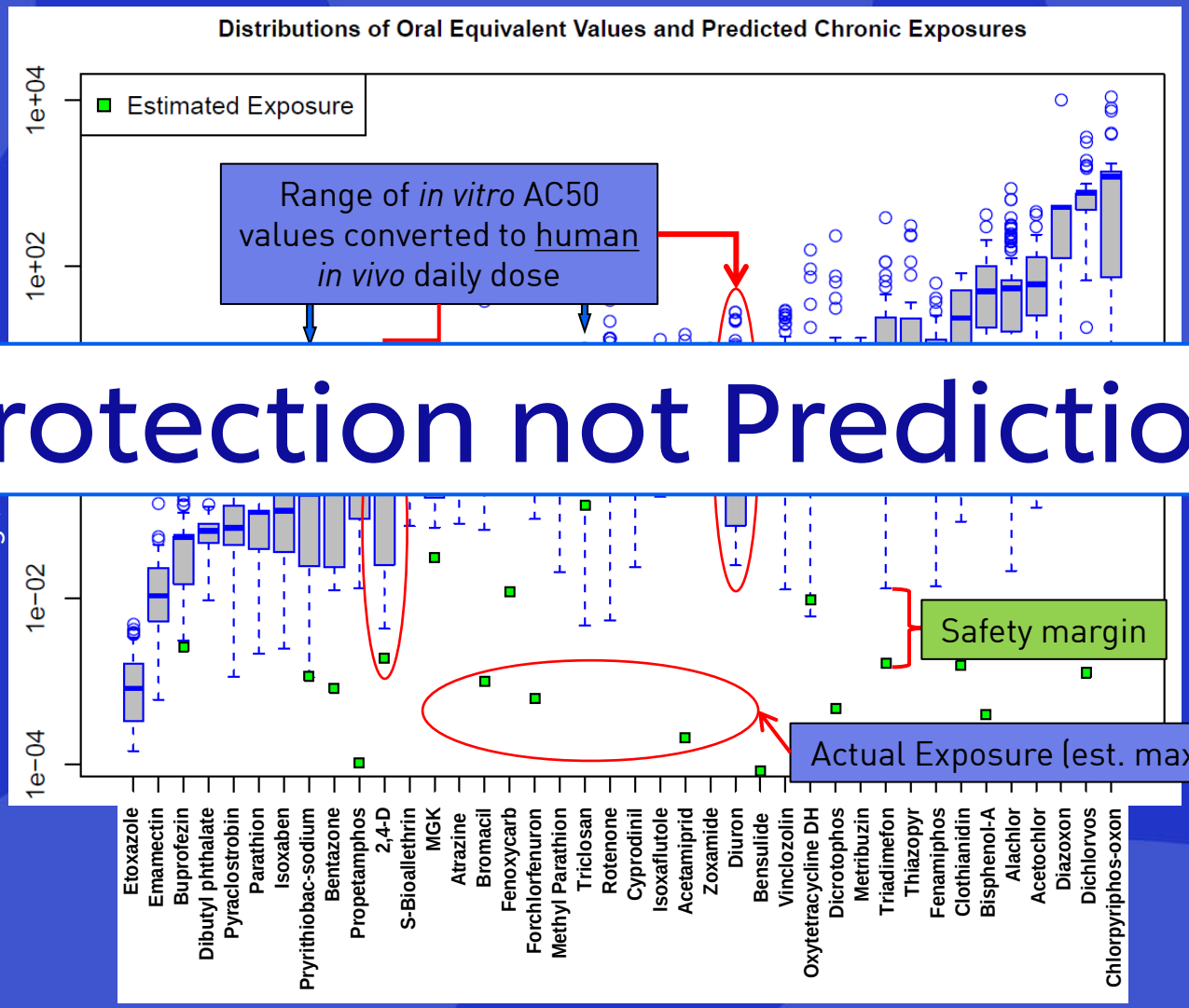
National Institute of Environmental Health Sciences (NIEHS) / National Toxicology Program (NTP)

National Center for Advancing Translational Sciences (NCATS)

U.S. Food and Drug Administration (FDA)

National Center for Computational Toxicology (EPA)

In Vitro Bioactivity vs Bioavailability



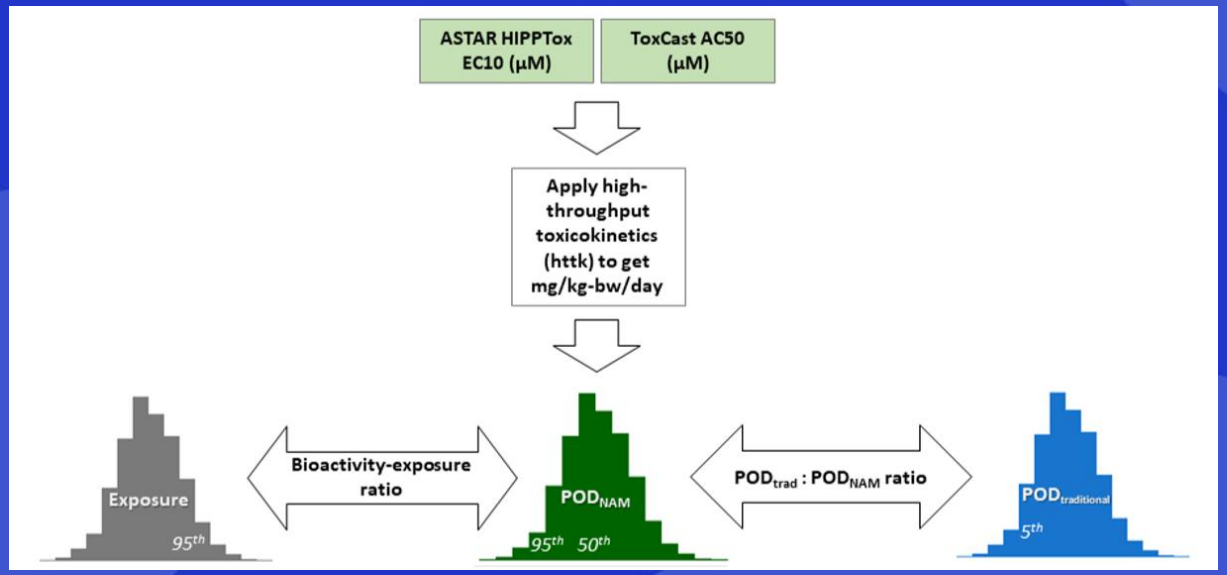
"Protection not Prediction"

Hepatic clearance and plasma protein binding determinations

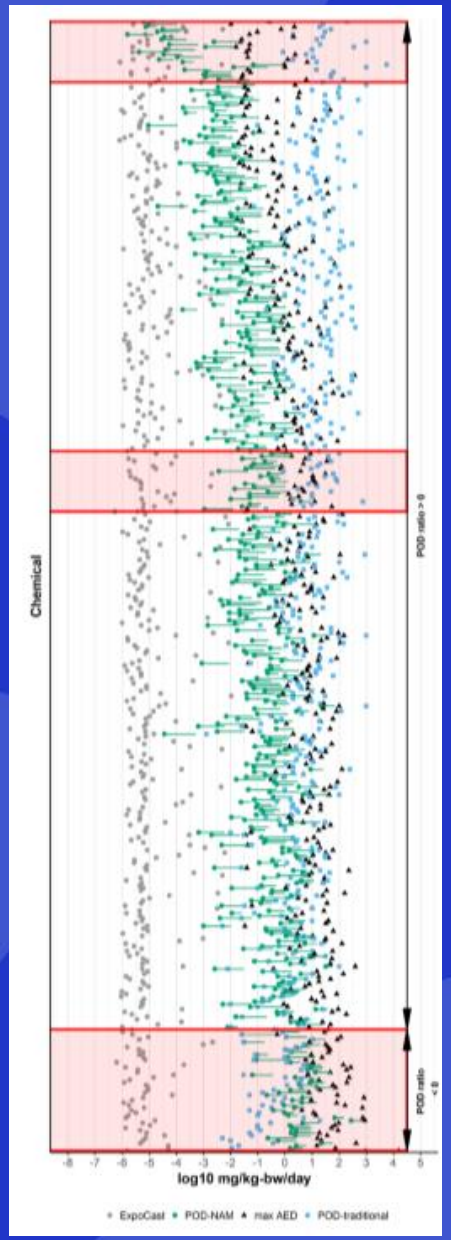
Slide from Dr Rusty Thomas, EPA, with thanks

Rotroff, *et al.* Tox.Sci 2010

EPA, NTP, HC, A*STAR, ECHA, EFSA, JRC, RIVM…

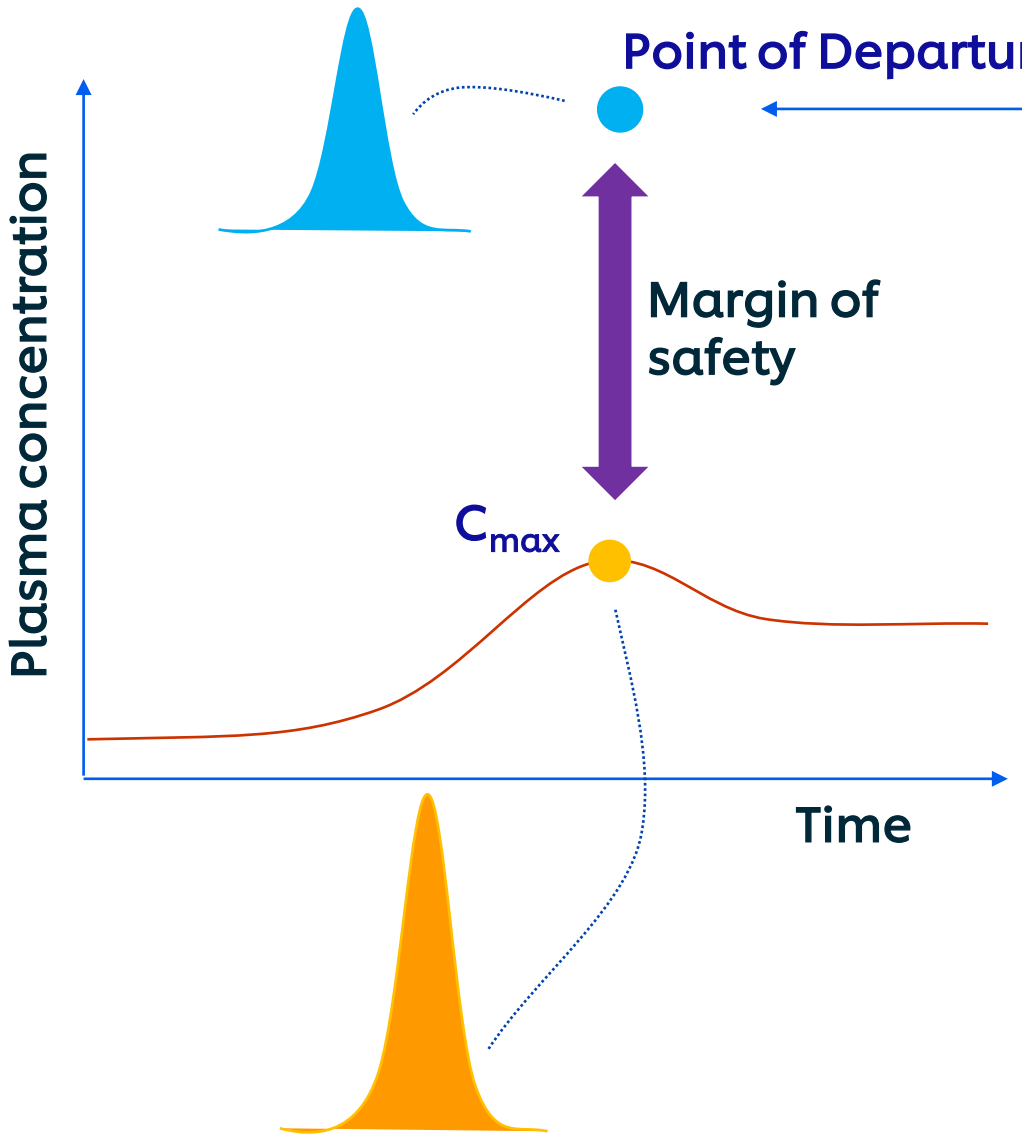


Katie Paul-Friedman *et al.* 2019 *Tox Sci* 173(1): 202-225

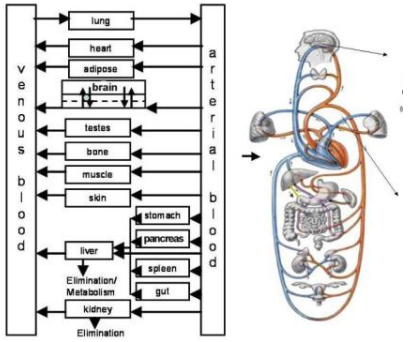


414/448 chemicals =
92% of the time this naïve approach appears conservative

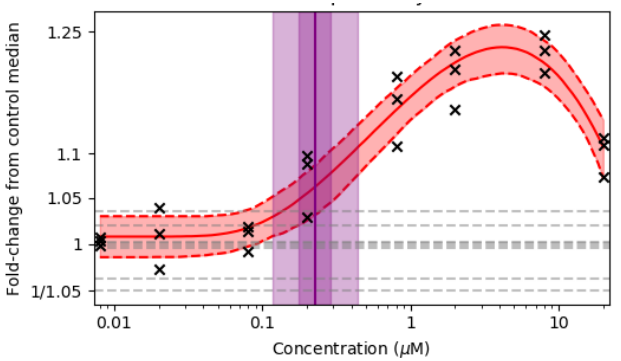
The Margin of Safety Approach



Exposure models (PBK, free/total concentration)



Point of departure derived from *in vitro* concentration-response



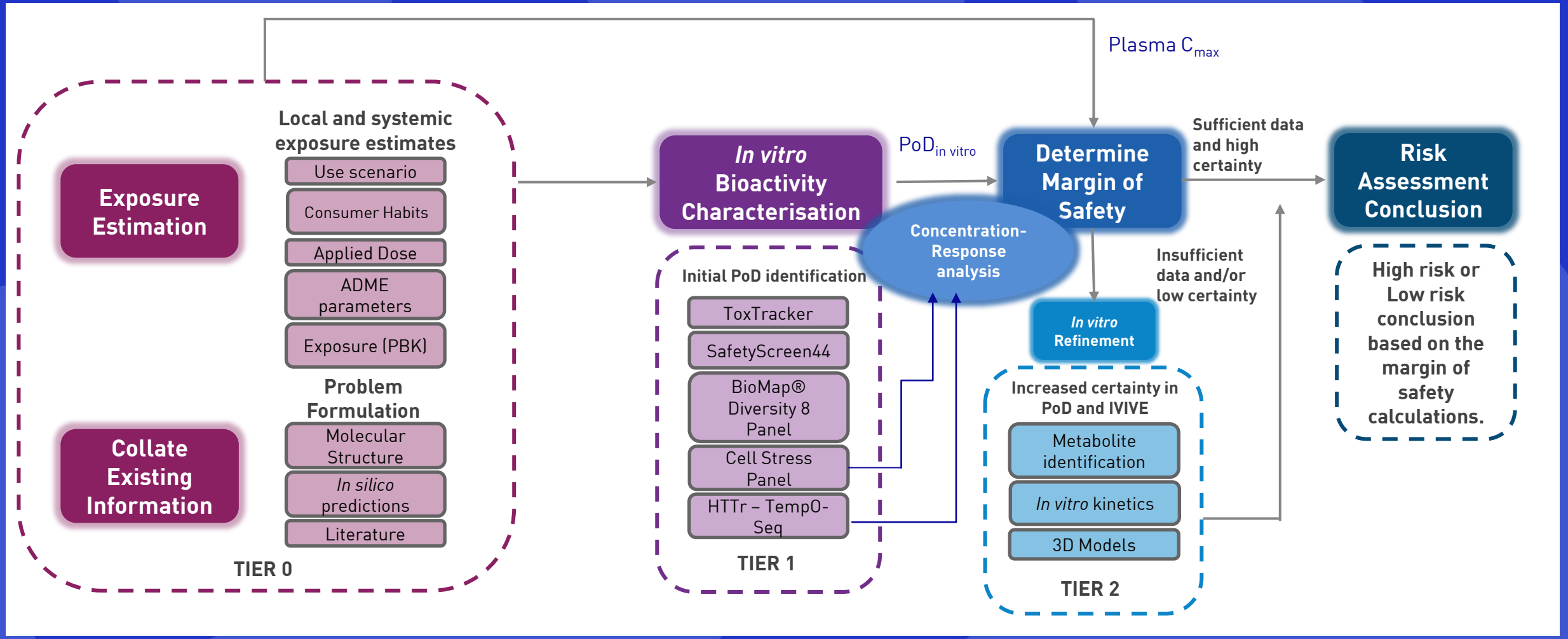
Case Study Approach… Imagine we have no data for: Coumarin

Safety assessment required for 0.1% coumarin in Body Lotion

Safety assessment required for 0.1% coumarin in Face Cream

Baltazar *et al.*, (2020) *Tox Sci* (in press) https://doi.org/10.1093/toxsci/kfaa048

Ab Initio NGRA Framework



Baltazar *et al.*, (2020) *Tox Sci* (in press) https://doi.org/10.1093/toxsci/kfaa048

Collection of Existing Data and ADME Parameters

Name	Coumarin
CAS	91-64-5
MW	146.14 g/mol
Log P	1.39
Solubility	0.96 mg/mL in phosphate buffer
ECCS Class	Class 2 (Metabolism)
R_{b2p}	0.7
F_{ub}	0.31
Cl_{int}	929 L/h

Chemistry determinations:

- Partition coefficient logP
- Peptide binding potential

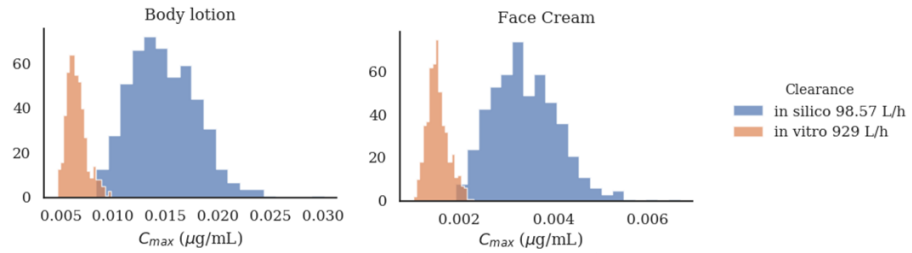
In vitro determined:

- Kinetic solubility
- Thermodynamic solubility
- Metabolic & chemical stability
- Stability in human plasma
- Plasma protein binding
- Partitioning in blood
- Skin penetration parameters

Systemic Bioavailability using PBK Modelling

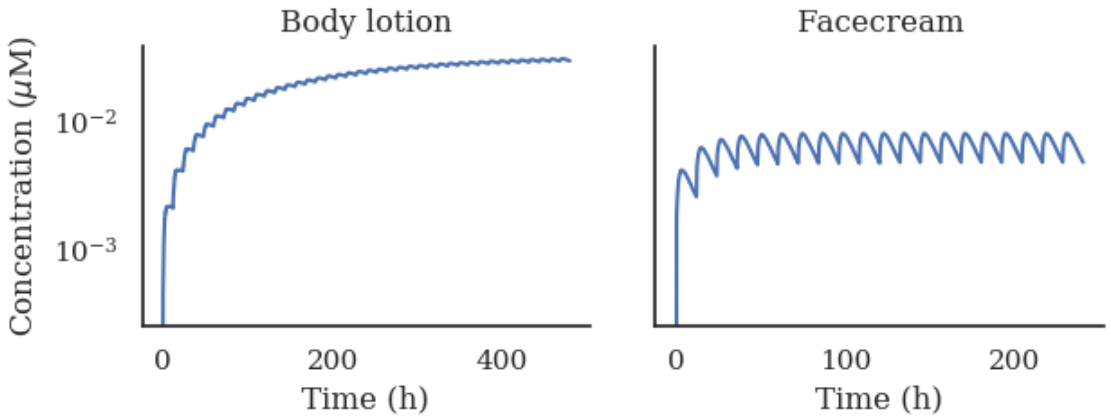
Key output parameters from uncertainty analysis:

Parameter	Face cream (applied 2x/day)	Body lotion (applied 2x/day)
Plasma Cmax total (µM)	0.023	0.10
95th percentile Cmax (µM)	0.032	0.14



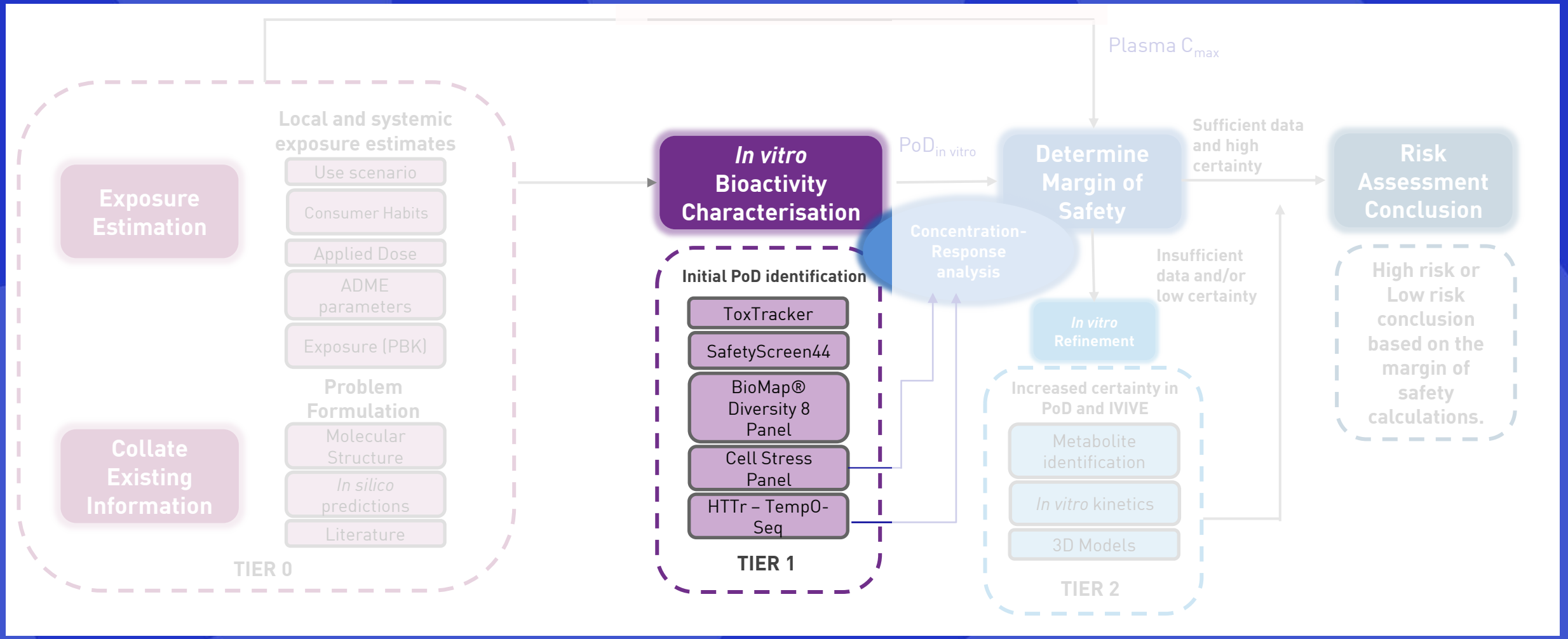
Uncertainty & Population Variability

0.1% Face cream & body lotion in Europe



Physiologically-based kinetic modelling using GastroPlus® v9.5. Estimations based on experimental data (Clint, fup, bpr, solubility, LogP). Skin penetration parameters were fitted against skin penetration data.

Ab Initio NGRA Framework

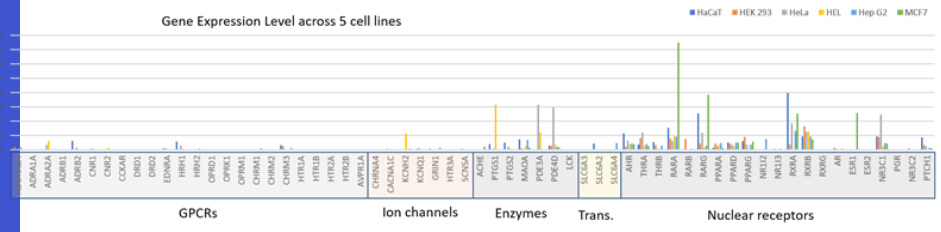


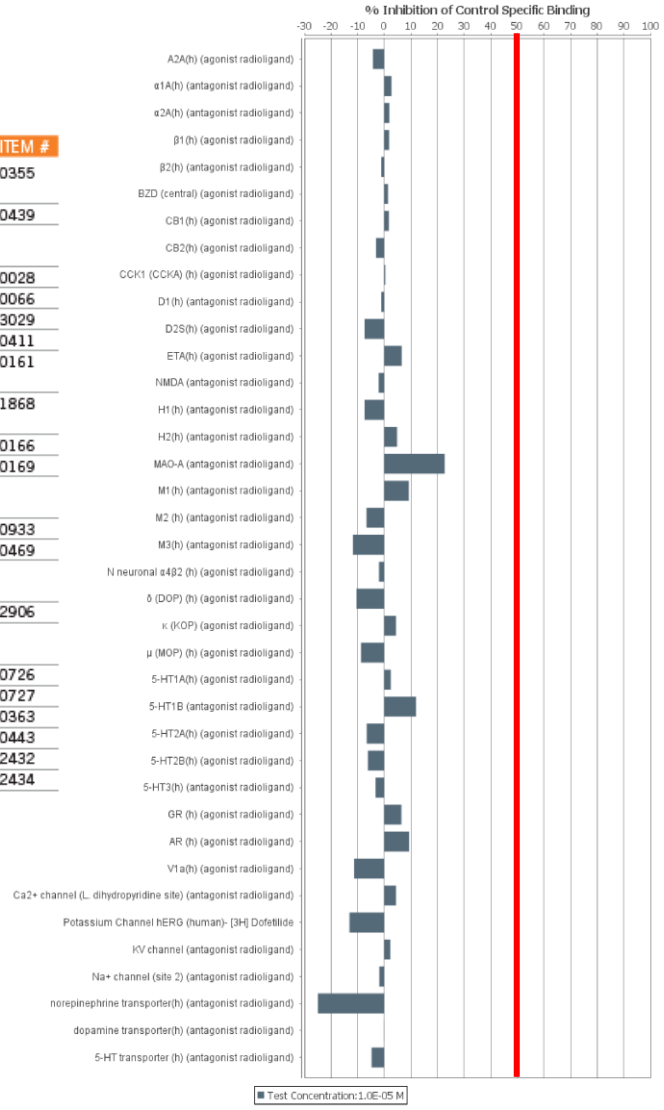
In Vitro Bioactivity: Safety Screen

eurofins | Cerep

Bowes et al 2012. Nature Reviews: Drug Discovery 11 909-922

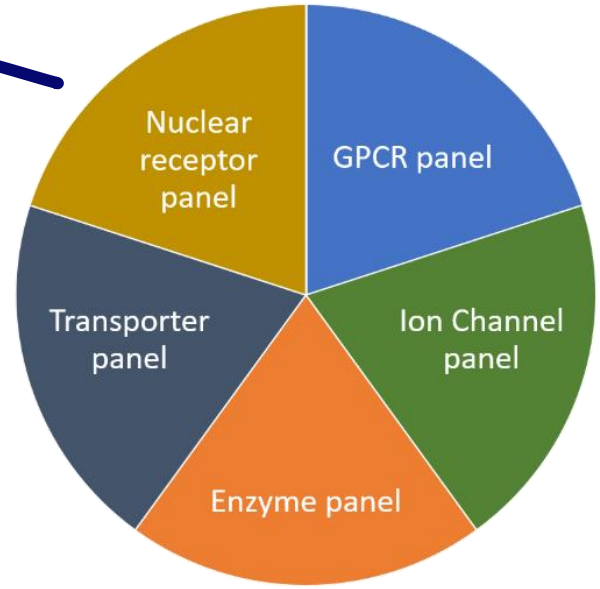
FAMILY	ASSAY	FORMAT	ITEM #
GPCR			
ADENOSINE	A_{3A}	•	0004
ADRENERGIC	$alpha_{1A}$		2338
	$alpha_{2A}$		0013
	$beta_1$	•	0018
	$beta_2$	•	0020
CANNABINOID	CB_1	•	0036
	CB_2	•	0037
CHOLECYSTOKININ	CCK_1 (CCK_A)	•	0039
DOPAMINE	D_1		0044
	D_{2S}	•	1322
ENDOTHELIN	ET_A	•	0054
HISTAMINE	H_1		0870
	H_2		1208
MUSCARINIC	M_1		0091
	M_2		0093
	M_3		0095
OPIOID & OPIOID-LIKE	$delta_2$ (DOP)	•	0114
	kappa (KOP)	•	1971
	mu (MOP)	•	0118
SEROTONIN	$5\text{-}HT_{1A}$	•	0131
	$5\text{-}HT_{1B}$		0132
	$5\text{-}HT_{2A}$	•	0471
	$5\text{-}HT_{2B}$	•	1333
VASOPRESSIN	V_{1a}	•	0159
TRANSPORTERS			
DOPAMINE	dopamine transporter		0052
NOREPINEPHRINE	norepinephrine transporter		0355
SEROTONIN	5-HT transporter		0439
ION CHANNELS			
GABA CHANNELS	BZD (central)	•	0028
GLUTAMATE CHANNELS	NMDA		0066
NICOTINIC CHANNELS	N neuronal α4β2	•	3029
SEROTONIN CHANNELS	$5\text{-}HT_3$		0411
Ca^{2+} CHANNELS	Ca^{2+} channel (L, dihydropyridine site)		0161
K^+ CHANNELS	hERG (membrane preparation)		1868
	K_v channel		0166
Na^+ CHANNELS	Na^+ channel (site 2)		0169
NUCLEAR RECEPTORS			
STEROID NUCLEAR RECEPTORS	AR	•	0933
	GR	•	0469
KINASES			
CTK	Lck kinase		2906
OTHER NON-KINASE ENZYMES			
AA METABOLISM	COX_1		0726
	COX_2		0727
MONOAMINE & NEUROTRANSMITTER	acetylcholinesterase		0363
	MAO-A		0443
PHOSPHODIESTERASES	PDE3A		2432
	PDE4D2		2434





All binding and enzymatic assay results were negative at 10 uM

No receptor/target-led pharmacological effect



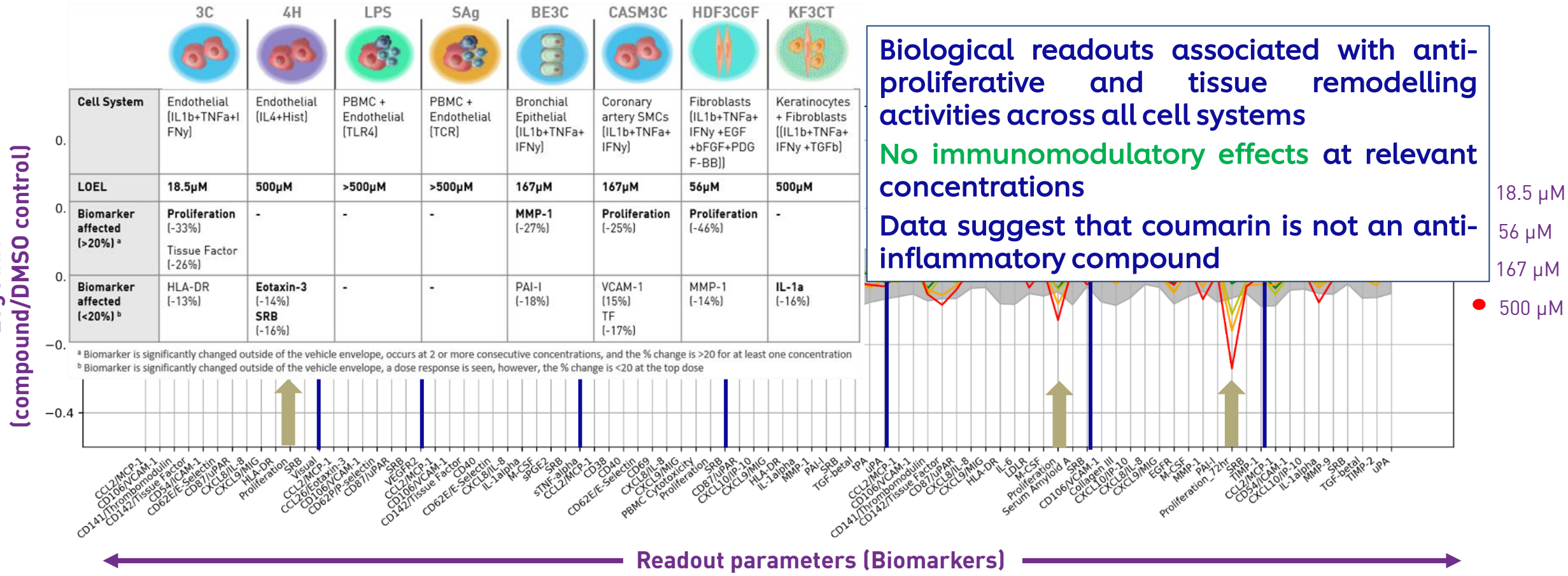
SafetyScreen44™ Panel

Immunomodulatory Bioactivity: BioMap® Diversity 8 Panel

BioMAP systems contain human primary cell types (or combinations) that are stimulated to replicate complex cell and pathway interactions of vascular inflammation, immune activation and tissue remodelling

	3C	4H	LPS	SAg	BE3C	CASM3C	HDF3CGF	KF3CT
Cell System	Endothelial (IL1b+TNFa+IFNy)	Endothelial (IL4+Hist)	PBMC + Endothelial (TLR4)	PBMC + Endothelial (TCR)	Bronchial Epithelial (IL1b+TNFa+IFNy)	Coronary artery SMCs (IL1b+TNFa+IFNy)	Fibroblasts (IL1b+TNFa+IFNy +EGF +bFGF+PDGF-BB))	Keratinocytes + Fibroblasts ((IL1b+TNFa+IFNy +TGFb)
LOEL	**18.5µM**	**500µM**	**>500µM**	**>500µM**	**167µM**	**167µM**	**56µM**	**500µM**
Biomarker affected (>20%) [a]	**Proliferation** (-33%) Tissue Factor (-26%)	-	-	-	**MMP-1** (-27%)	**Proliferation** (-25%)	**Proliferation** (-46%)	-
Biomarker affected (<20%) [b]	HLA-DR (-13%)	**Eotaxin-3** (-14%) **SRB** (-16%)	-	-	PAI-I (-18%)	VCAM-1 (15%) TF (-17%)	MMP-1 (-14%)	**IL-1a** (-16%)

[a] Biomarker is significantly changed outside of the vehicle envelope, occurs at 2 or more consecutive concentrations, and the % change is >20 for at least one concentration
[b] Biomarker is significantly changed outside of the vehicle envelope, a dose response is seen, however, the % change is <20 at the top dose



Biological readouts associated with anti-proliferative and tissue remodelling activities across all cell systems

No immunomodulatory effects at relevant concentrations

Data suggest that coumarin is not an anti-inflammatory compound

In Vitro Bioactivity: Cell Stress Panel

Hatherall et al., 2020 *Tox Sci* (Accepted)

~40 Biomarkers; 3 Timepoints; 8 Concentrations; ~10 Stress Pathways

Step 1

Selection of stress pathways

Mitochondrial Toxicity , Oxidative Stress, DNA damage, Inflammation, ER Stress, Metal Stress, Heat Shock, Hypoxia, Cell Health

Selection of biomarkers, probes or antibodies and optimisation of high-content imaging

Selection of cell line, exposure scenario and timepoints

HepG2 cell line, single exposure, 1h, 6h and 24h

Step 2

Selection of chemicals according to different classes and exposure scenarios (based on typical use of compound)

Non-stress inducers

- Caffeine (beverages, cosmetics)
- Coumarin (food, cosmetics)
- Niacinamide (food, cosmetics)
- Phenoxyethanol (cosmetics)

Stress inducers

- CDDO-Me (drug)
- Sulforaphane (food)
- DEM (industrial chemical)
- tBHQ (antioxidant)
- Doxorubicin (drug)
- Diclofenac (drug)
- Triclosan (antimicrobial)
- Troglitazone (drug)
- Pioglitazone (drug)
- Rosiglitazone (drug)

Step 3

Selection of *in vitro* concentrations based upon realistic human exposures

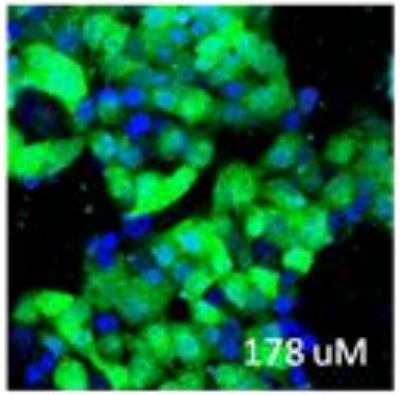
Information on human exposure obtained from human clinical trials or PBK modelling

Selection of 8 *in vitro* concentrations (upper bound limited by ~20% cytotoxicity

Key

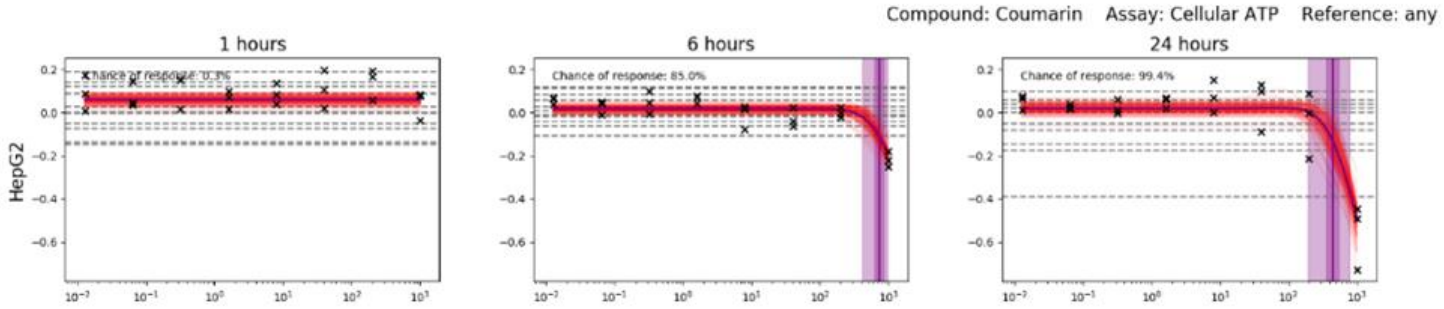
- Exposure scenario adopted for chemical is 'high risk' (from consumer goods perspective).
- Exposure scenario adopted for chemical is 'low risk' (from consumer goods perspective).

- Mitochondrial Toxicity
- Oxidative Stress
- DNA damage
- Inflammation
- ER Stress
- Metal Stress
- Osmotic Stress
- Heat Shock
- Hypoxia
- Cell Health



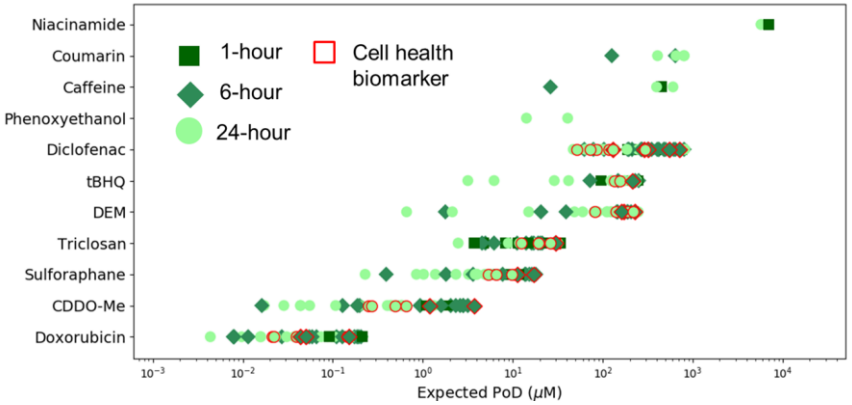
*now conducted in HepaRG spheroids

In Vitro Bioactivity: Cell Stress Panel



Biomarker	Stress pathway	PoD (2.5th percentile), µM	PoD (50th percentile), µM	PoD (97.5th percentile), µM	Effect
Cell count (72h)	Cell health	54	150	316	down
ATP (6h) **ATP (24h)**	Cell health	411 194	738 449	976 763	down
GSH (24h)	Oxidative stress	641	781	979	up
IL-8 (6h) **IL-8 (24H)**	Inflammation	8.8 343	52 698	123 974	down
Phosholipidosis (24h) **Phosholipidosis (72h)**	Cell health	289 285	605 588	949 915	down
LDH (1h)	Cell health	52	370	974	up
ICAM-1 (24h)	Inflammation	354	696	973	down
Steatosis	Cell health	59	659	974	up

Summary with PoD for cell stress biomarkers:



- Coumarin not very active in comparison to known 'high risk compounds' like doxorubicin, diclofenac etc.
- Cell count, cellular ATP, GSH, IL-8, Phospholipids, LDH, ICAM-1 and steatosis showed a dose response

In Vitro Bioactivity: Tempo-Seq Technology

High-Throughput Transcriptomics Gene Expression Profiling (HTTr)

1. Defining a safe operating exposure for systemic toxicity using a **NOTEL** (No Transcriptional Effect Level)
2. Defining compound similarity grouping (Read Across)

NOTEL is the derived concentration of a compound that does not elicit a meaningful change in gene expression (i.e. the threshold of the concentration that elicits minimal mechanistic activity)

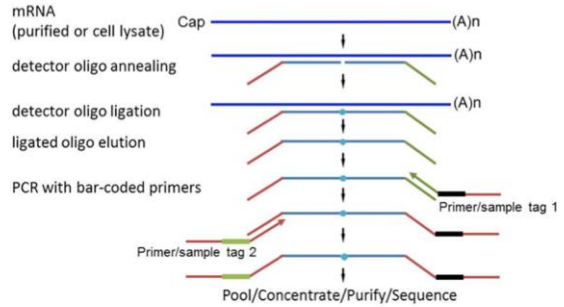
Cell lines (chosen to express a range of relevant receptors)

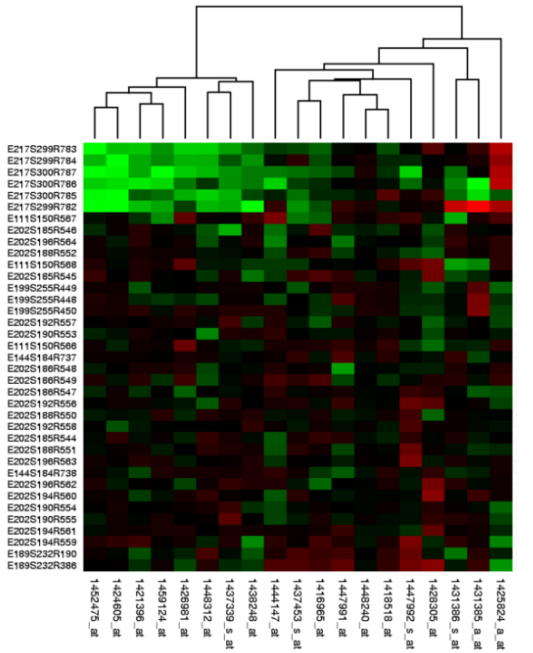
MCF-7 – human breast adenocarcinoma cell line

HepG2 – human liver carcinoma

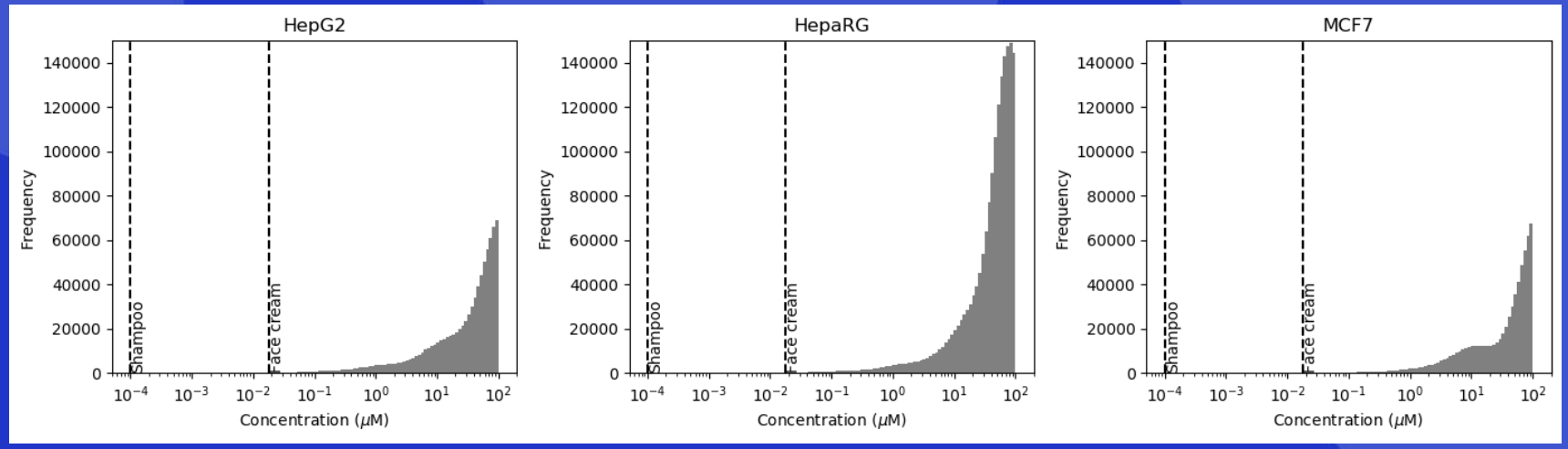
HepaRG – terminally differentiated hepatic cells that retain many characteristics of primary human hepatocytes + as spheroids

N-HEK – primary normal human epidermal keratinocytes





In Vitro Bioactivity: Tempo-Seq Technology



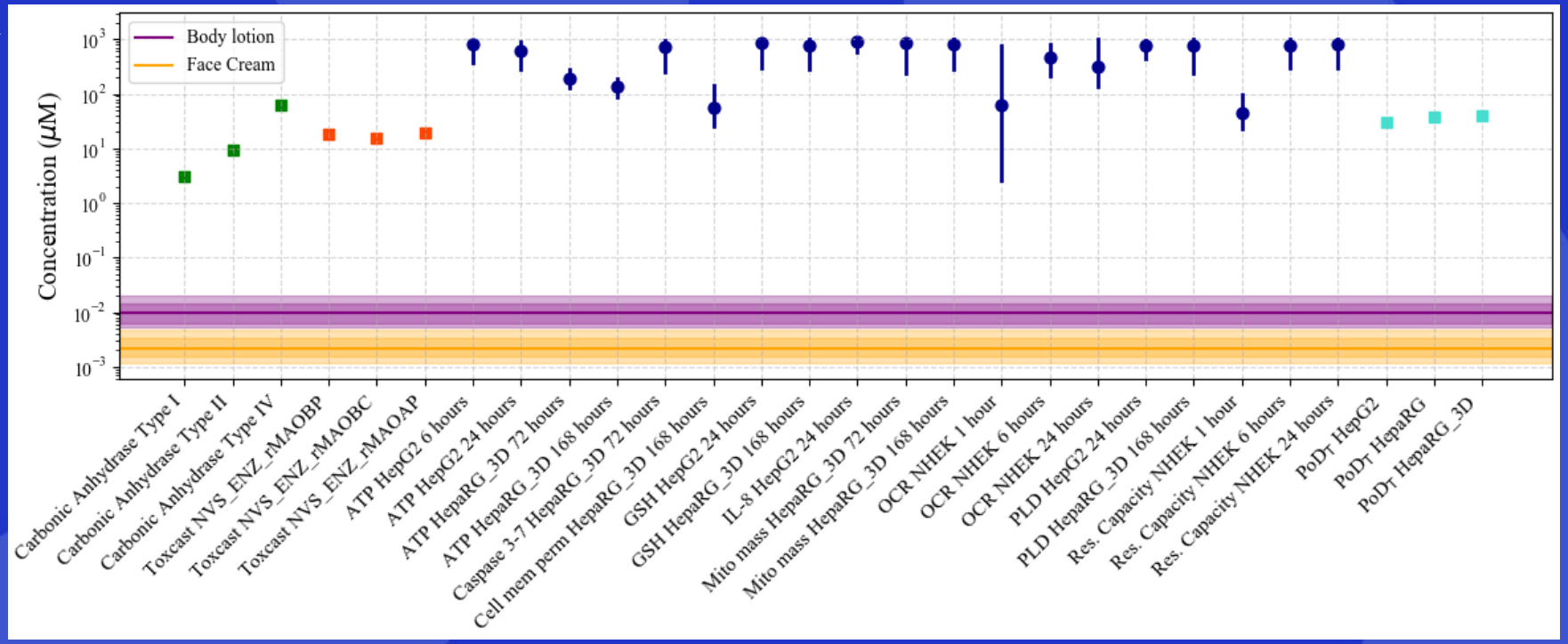
- Coumarin dose range 0.001uM to 100uM
- 24 hour time point
- QC and normalisation in DESeq2
- BMDExpress2 applied to determine NOTEL
  (3 pathway approaches)

Cell Model	HepG2	MCF7	HepaRG 2D
Pathway Level Tests	(308 pathways)	(0 pathways)	(17 pathways)
20 pathways with the lowest pvalue Reactome	70	NA	58*
20 pathways with the lowest BMD Reactome	44	NA	58*
BMD of Reactome pathway with lowest BMD that meets significance threshold criteria	31	NA	38
Gene Level Tests	(1570 genes)	(47 genes)	(87 genes)
Mean BMD of 20 genes with largest fold change	6	3	54
Mean BMD of Genes between 25th and 75th percentile	17	1	59

Margin of Safety considering PODs and Exposure

PoDs and plasma C_{max} (μM) are expressed as total concentration

PubChem **ToxCast** **Cell Stress Panel** **HTTr**



C_{max} expressed as a distribution:

- Line = median (50th percentile)
- Inner band = 25th-75th percentile
- Outer band = 2.5th-97.5th percentile (95th credible interval)

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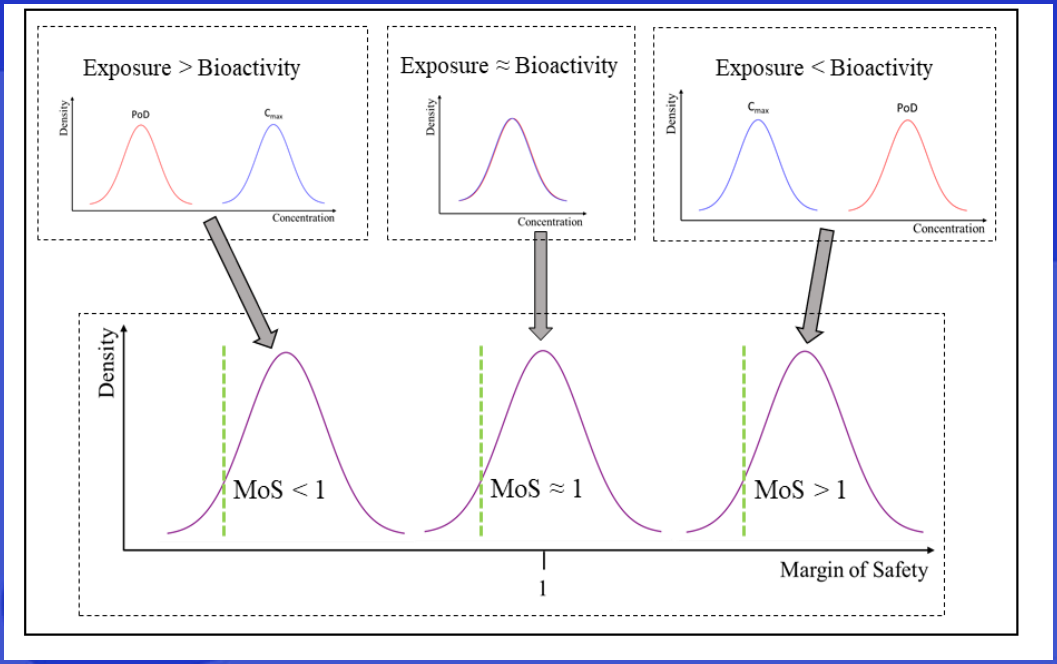
Application of *Ab Initio* Approach: Risk Assessment (NGRA)

Margin of safety is the **fold difference** between the **Cmax** and the ***in vitro*** **POD**



Technology	Cell line/ Enzyme/Biomarker	Face cream Min. 5th percentile MoS	Body Lotion Min. 5th percentile MoS
Cell stress panel	HepG2 (ATP, 24h)	96738	22048
Cell stress panel	NHEK (OCR 1h)	1330	**295**
HTTr	HepG2 (24h)	7223	1618
HTTr	HepaRG (24h)	8864	1986
Toxcast	MAO B	3711	831
PubChem	Carbonic Anhydrase Type I	**706**	**158**
PubChem	Carbonic Anhydrase Type II	2140	479
PubChem	Carbonic Anhydrase Type VI	14652	3282
Cell stress panel	HepaRG_3D (cell mem perm 168h)	9601	2197
HTTr	HepaRG_3D_24h	9538	2137

Unilever

Ab Initio NGRA Framework

Plasma C_{max}

Exposure Estimation

Local and systemic exposure estimates

- Use scenario
- Consumer Habits
- Applied Dose
- ADME parameters
- Exposure (PBK)

Collate Existing Information

Problem Formulation

- Molecular Structure
- *In silico* predictions
- Literature

TIER 0

***In vitro* Bioactivity Characterisation**

$PoD_{in\ vitro}$

Initial PoD identification

- ToxTracker
- SafetyScreen44
- BioMap® Diversity 8 Panel
- Cell Stress Panel
- HTTr – TempO-Seq

TIER 1

Concentration-Response analysis

Determine Margin of Safety

Sufficient data and high certainty

Risk Assessment Conclusion

Insufficient data and/or low certainty

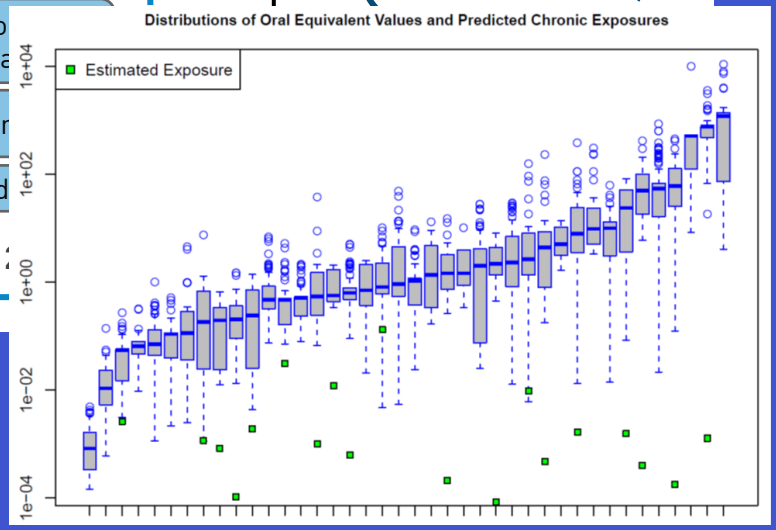
***In vitro* Refinement**

Increased certainty in PoD and IVIVE

- Metabo identifica
- *In vitro* kir
- 3D Mod

TIER 2

High risk or Low risk conclusion based on the margin of safety calculations.



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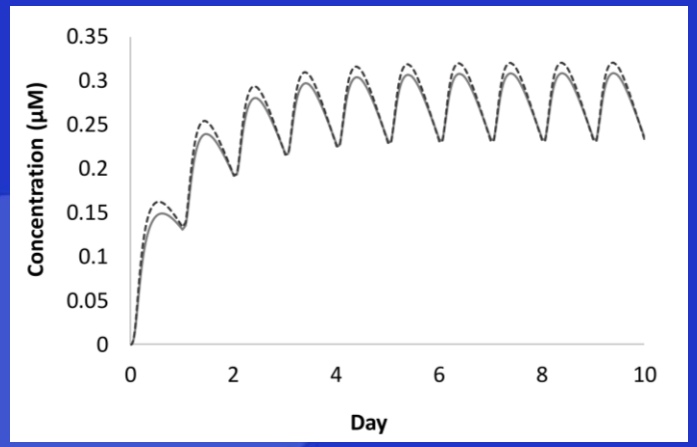
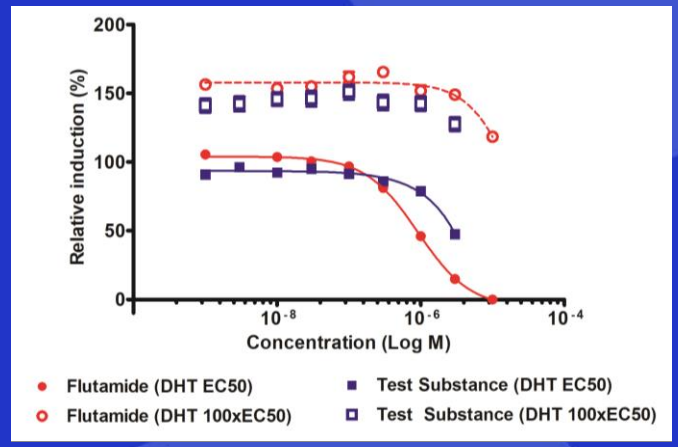
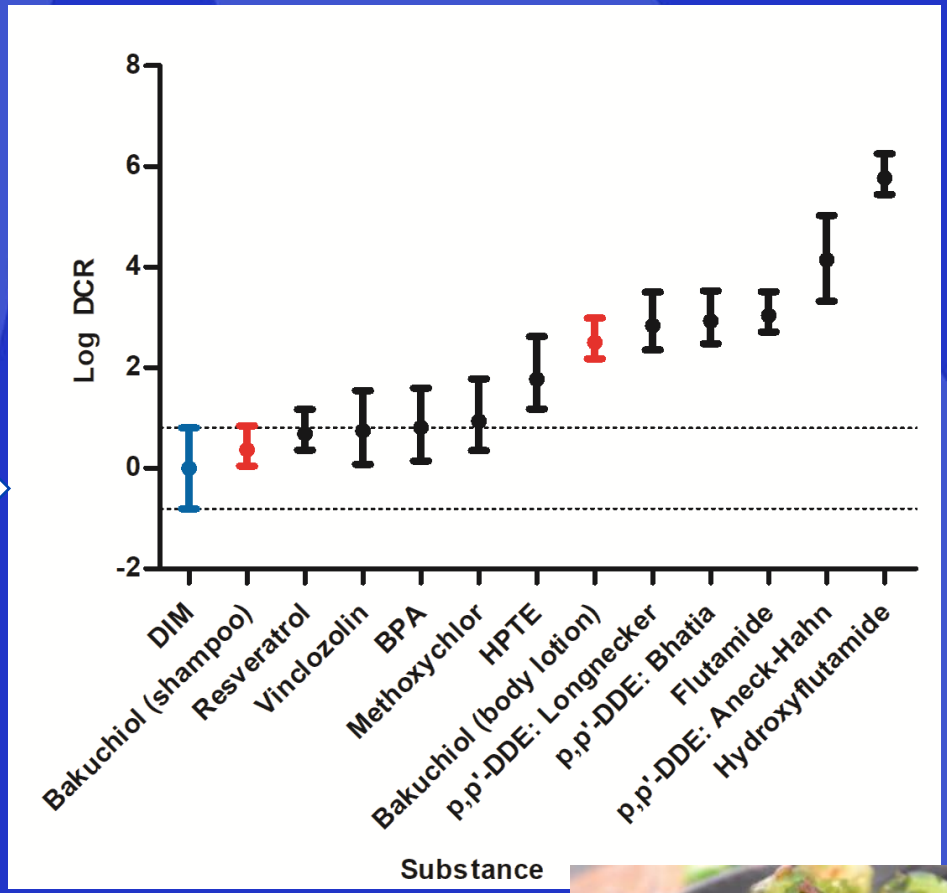
Making sense of margins of safety by benchmarking

Dent *et al.*, (2019) *Tox Sci* 167(2): 375-384

Exposure + Bioactivity data (substance and comparators)





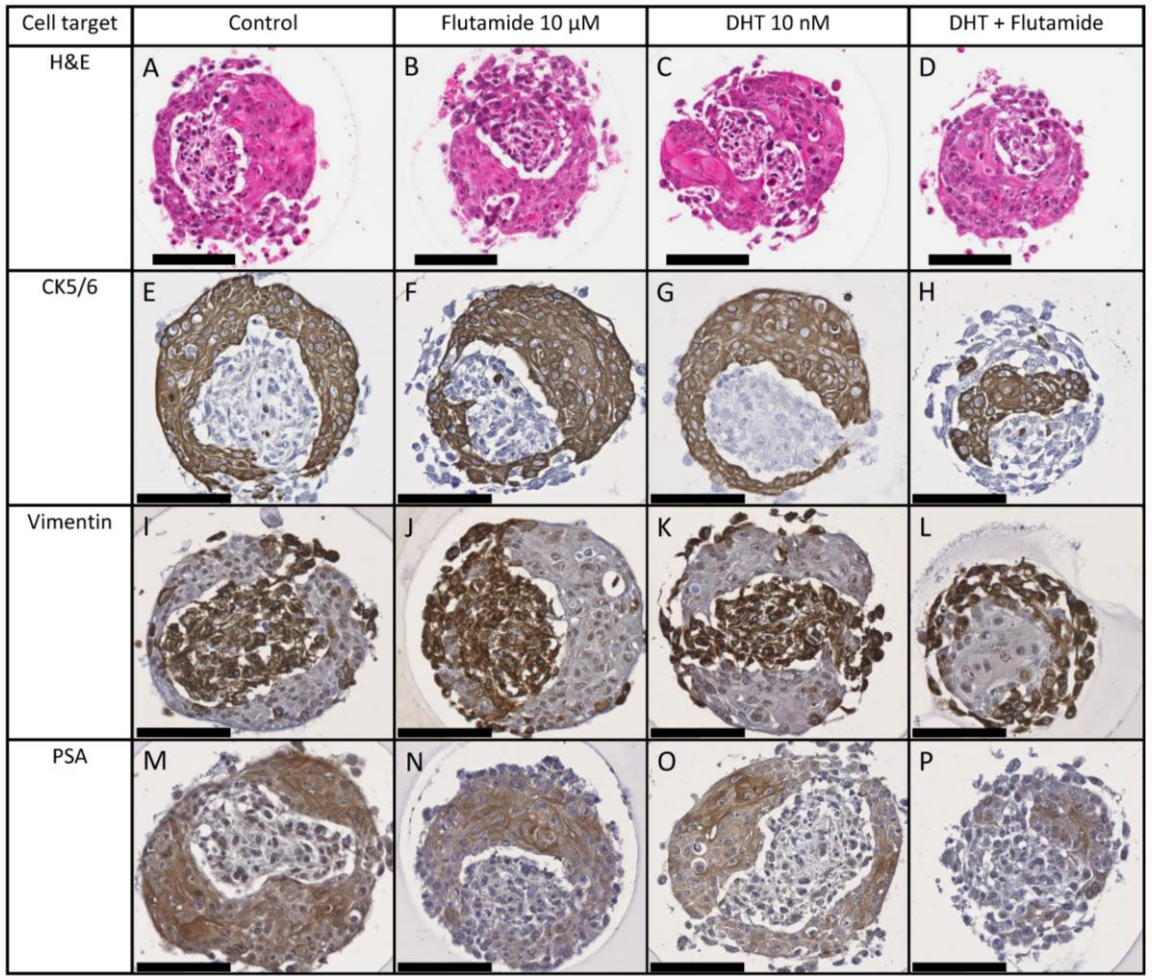


$$\text{EAR (unitless)} = \frac{\text{Exposure (plasma exposure in µM)}}{\text{Activity } (IC_{50}\ \mu M)}$$

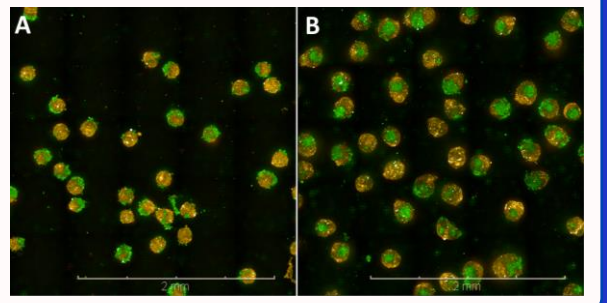
$$\text{DCR} = \frac{\text{EAR (test substance)}}{\text{EAR (dietary comparator)}}$$

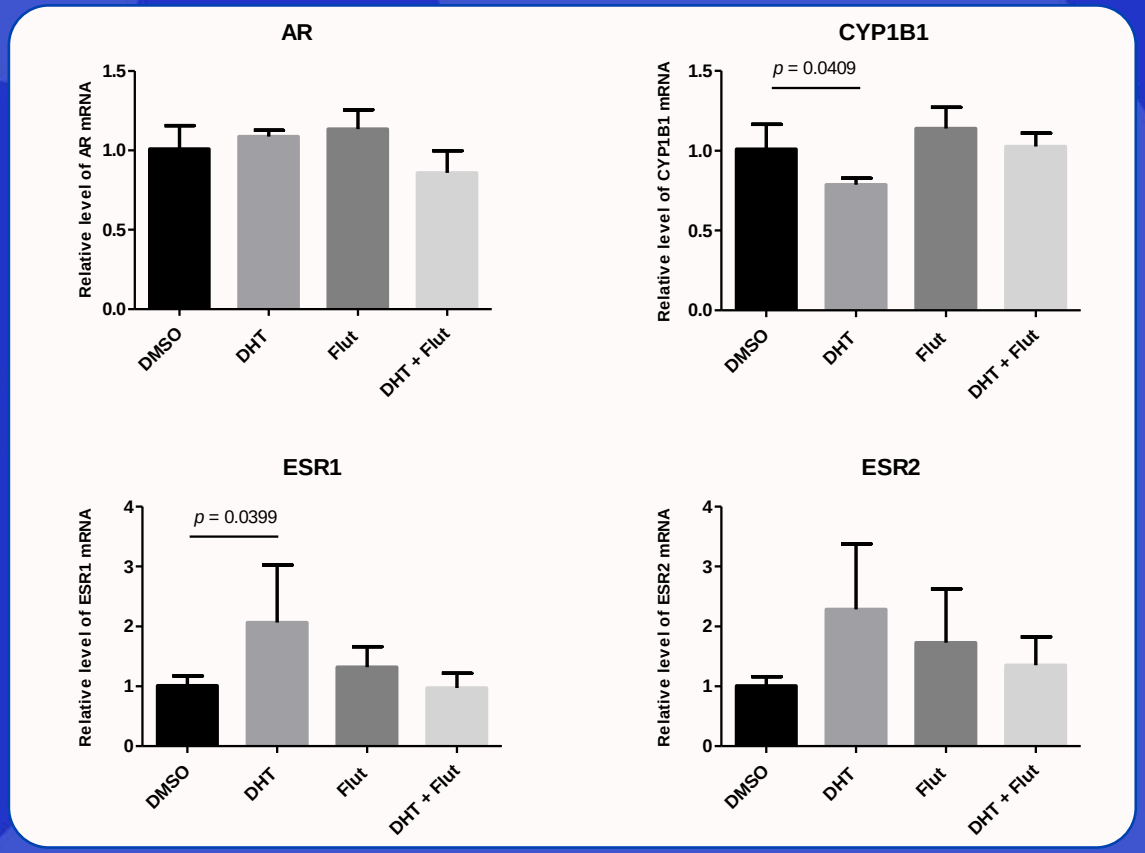
(After Becker *et al.*, (2015) *Regul Toxicol Pharmacol* 71(3): 398–408)

Higher tier tools to differentiate between activity and adversity



Cell cultures with more *in vivo* relevance + morphological and molecular biomarkers





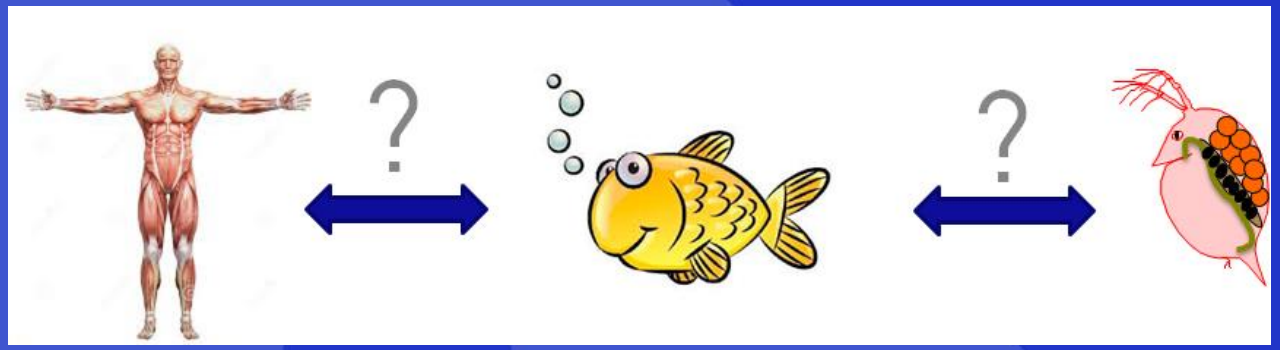
BROWN

Dent *et al.*, (2019) *TIV* 60: 203-211

Building confidence in NGRA

- Need to ensure quality/robustness of the non-standard (non-TG) work and to characterise uncertainty to allow informed decision-making
  - Cell types, study designs, decision points
- This is a seismic shift in approach - dialogue is needed
- More research, creativity and examples needed to build confidence

Common frameworks



- Wealth of available, but unexploited data
- Opportunity for knowledge sharing across (eco)toxicology

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Vision of a near future: Bridging the human health–environment divide. Toward an integrated strategy to understand mechanisms across species for chemical safety assessment

Conclusions

- Consumers are demanding change
- This has spurred progress in the development of next generation risk assessments in the consumer products industry
- NGRA is exposure-led, hypothesis driven, and requires clear articulation of the risk assessment question
- Progress is only possible with a change in mindset (protection not prediction)
- Shortcomings will be addressed by current and future research and more case studies
- Principles apply equally to environmental safety assessment

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