Novel Approaches and Technologies to Assess Genotoxic Modes of Action

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Central Product Safety

Ensuring Safe Products

Agenda

- Introduction
- MoA via genomic biomarkers
  - Approach taken
  - Transcriptomics
    - Tgx-DDI biomarker (DNA Damage Inducing)
    - Connectivity mapping
  - ToxTracker
- Conclusions, part 1
- Pyrrozolidinly alkaloids – use of MoA to support potency ranking
- Conclusions, part 2 and open questions

Introduction

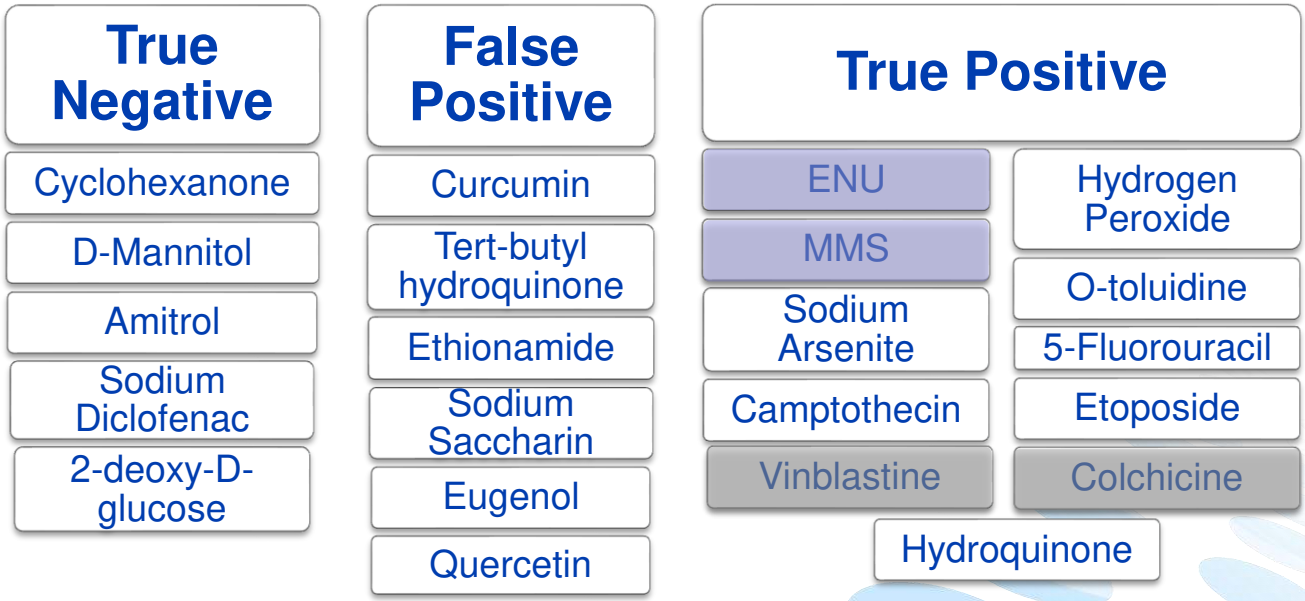
- P&G has a goal to develop/establish a reliable & predictive *in vitro* method for identifying mode of action (MoA)
- Initial interest is in disseminating primary (direct) from secondary (indirect) effects for its impact on risk assessment
- Approach taken is to compare different methodologies using genomic biomarkers that have been developed for classifying chemicals by MoA
  - Insights into what drives the toxic response
  - Draw conclusions regarding primary/secondary genotoxicity
  - Ultimate goal goes beyond classification
    - ➢ connect to data rich chemicals: read across
- Many different ways to approach MoA, other examples in WS
- Can support each other and reduce uncertainty

High burden of proof for regulatory decision making

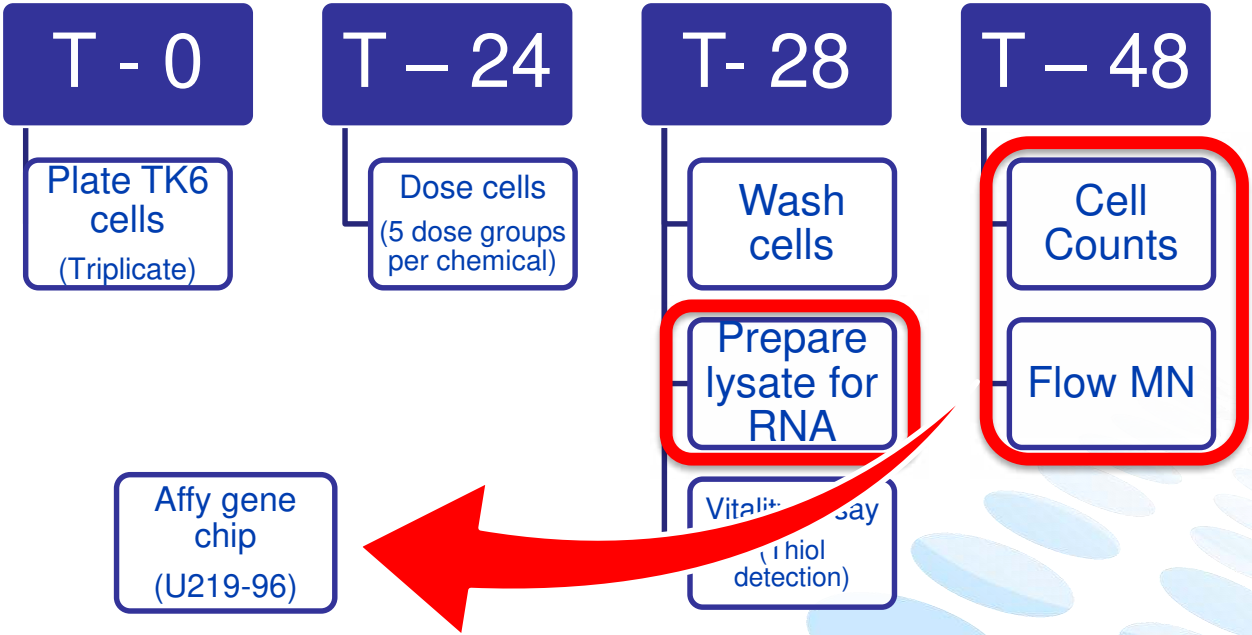
Approach

- Compare different methodologies that have been developed for classifying chemicals by MoA → TGx-DDI, C-Map, ToxTracker
- Examine the impact of the genomic platform used, and reduction of information
  - Test 22 chemicals using:
    - 'All-in-one approach' where samples from a relevant genotoxic endpoint (flow MN assay) are also used for genomic analysis (Affymetrix).
    - L1000 Expression Profiling **(Peck et al. Genome Biology 2006).** Uses "Landmark Genes" that reflect full genome expression profiles. (Cheaper, faster, more high throughput)
      - Analyze results and compare both using Connectivity Mapping (CMap)
    - Compare with results from coded testing with Toxtracker, a stem cell-based reporter assay

Selected Chemicals



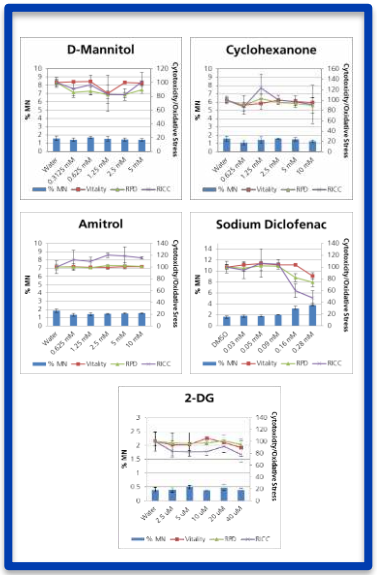
Combination Approach Overview



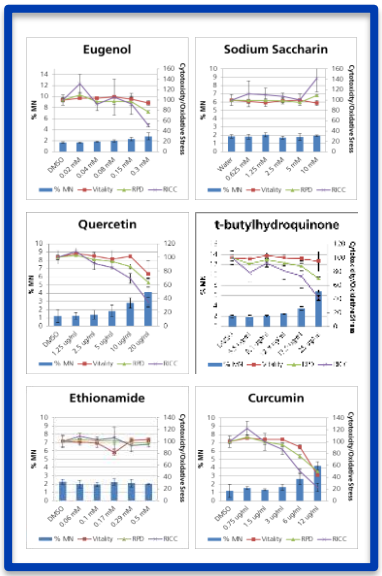
Results

1) Micronuclei in Tk6 cells

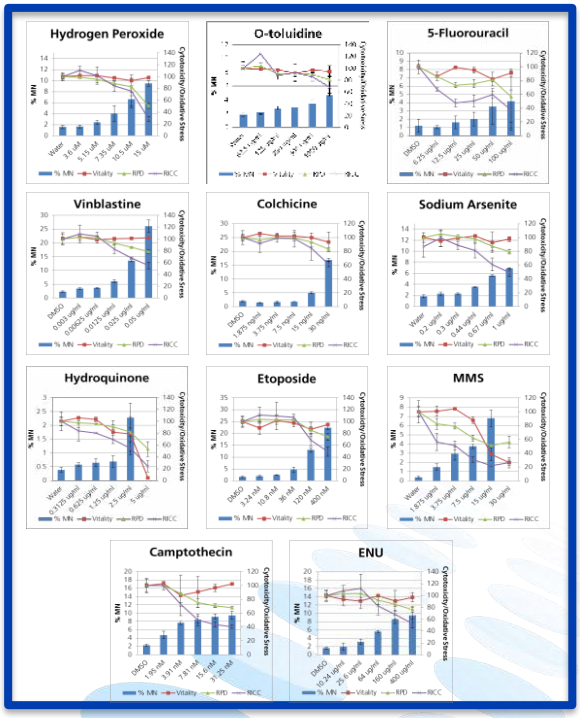
True Negative



False Positive

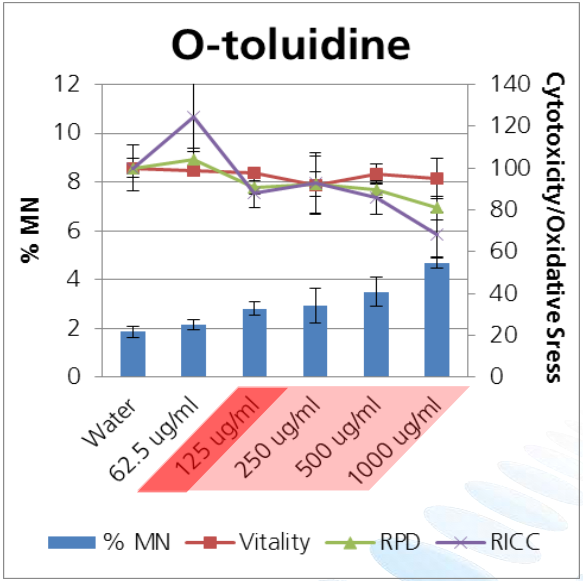


True Positive



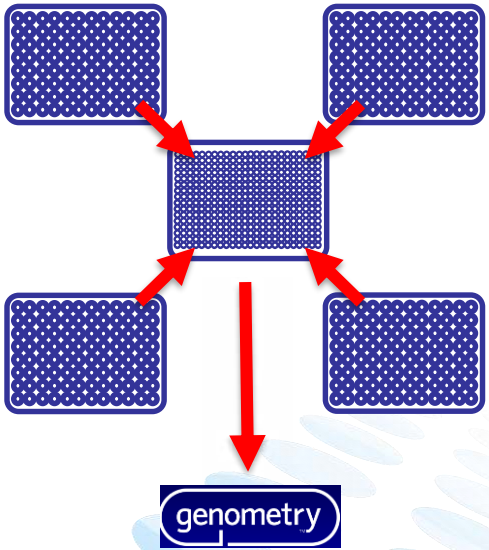
Dose selection for gene analysis

- **For Affy**
  - Cytotoxicity
  - MN response
  - Total no of affected genes
- **For CMap**
  - Selected single dose from Affy data
  - Moderate level of induction



L1000 Expression Profiling Overview

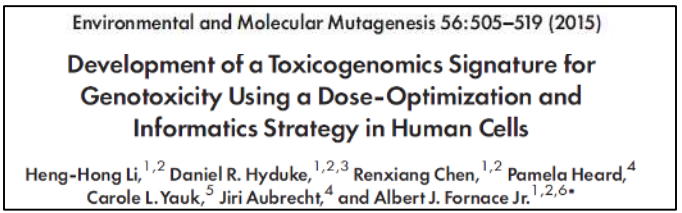
- TK6 cells were treated for 4 hours, then processed (crude lysates) and frozen at -80C.
- Selected 1-4 doses per compound using results from combination approach.
- 4 independent, randomized 96-well experiments performed.
- Transferred to a 384 well plate and sent to Genometry for analysis.



Data analysis 1 - TGx-DDI

- Result of a HESI Toxicogenomics team project
- Identification of DNA Damage Inducing (DDI) agents (no anuegens)
- Dose optimization protocol using qRT- PCR of stress response genes (CDKN1A; GADD45A; ATF3)
- Followed by microarray (Agilent) analysis
- 65-gene 'DDI' signature was determined

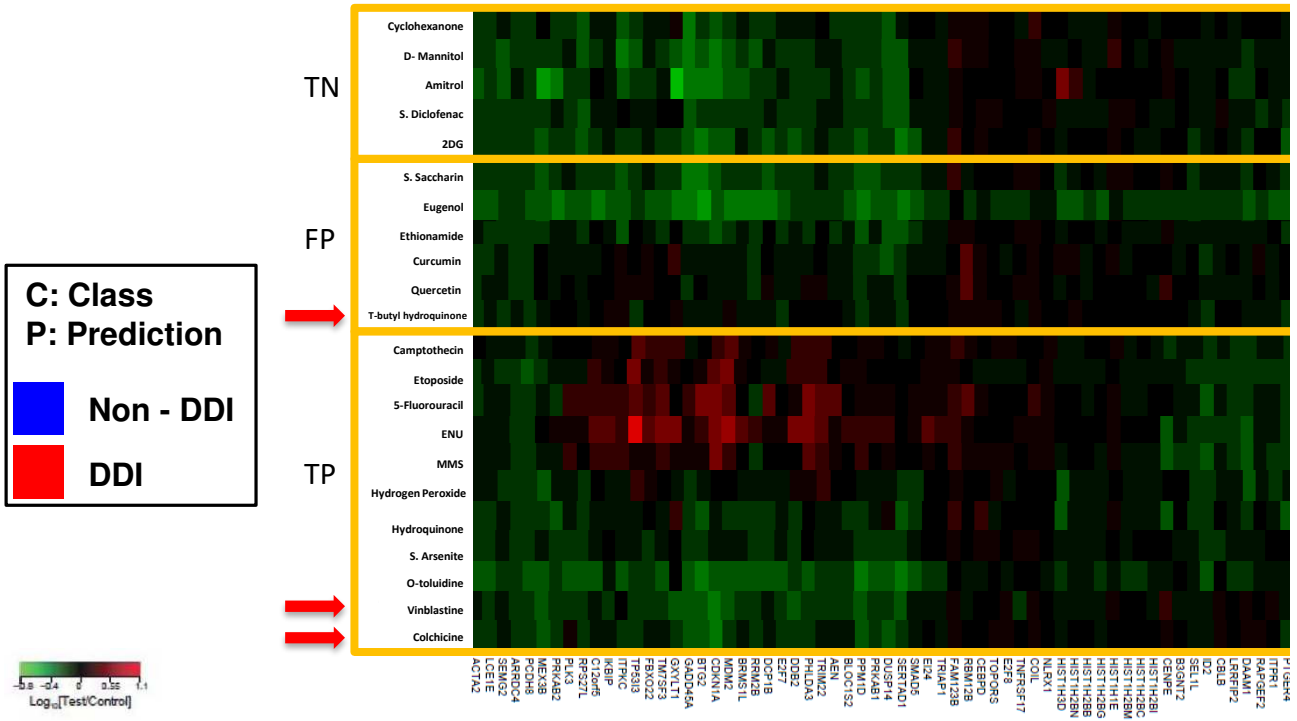
Our data were analyzed by Health Canada (Andrew Williams, Carole Yauk)


Environmental and Molecular Mutagenesis 56:505–519 (2015)

Development of a Toxicogenomics Signature for Genotoxicity Using a Dose-Optimization and Informatics Strategy in Human Cells

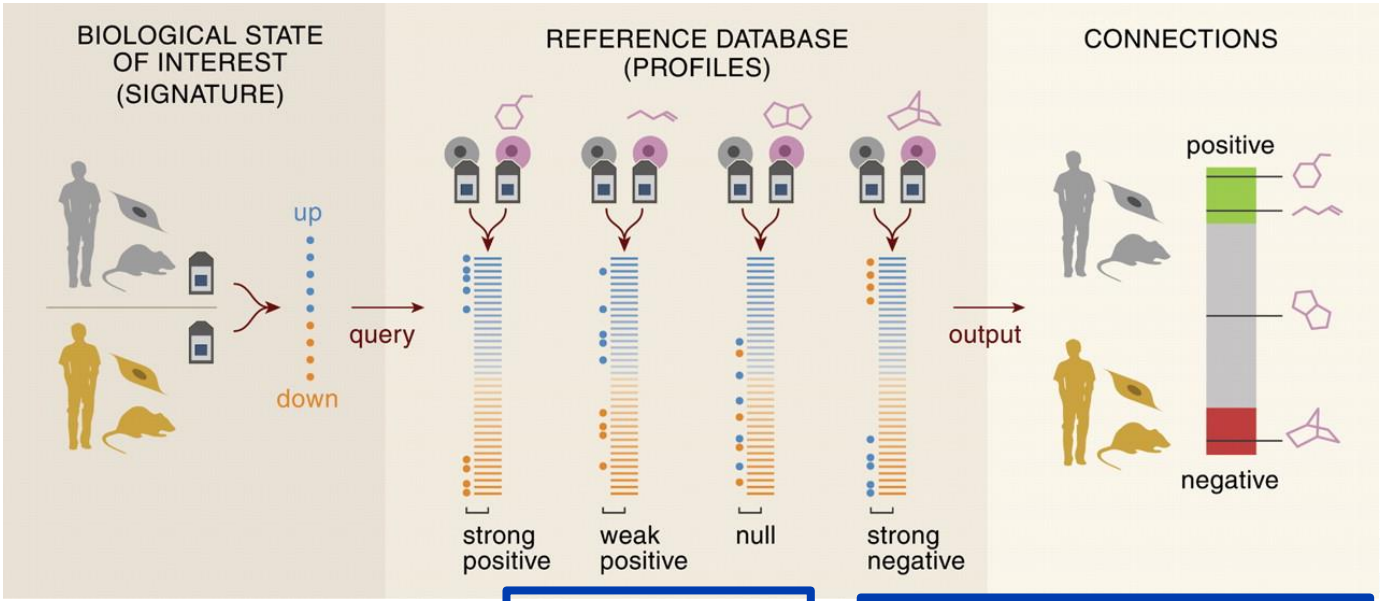
Heng-Hong Li,[1,2] Daniel R. Hyduke,[1,2,3] Renxiang Chen,[1,2] Pamela Heard,[4] Carole L. Yauk,[5] Jiri Aubrecht,[4] and Albert J. Fornace Jr.[1,2,6*]

TGx-DDI – 65 gene set

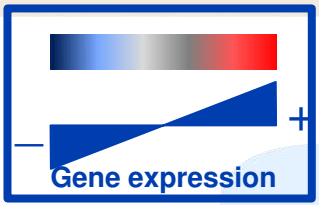


Data analysis 2: Whole genome information

The Connectivity Mapping (CMap) Concept

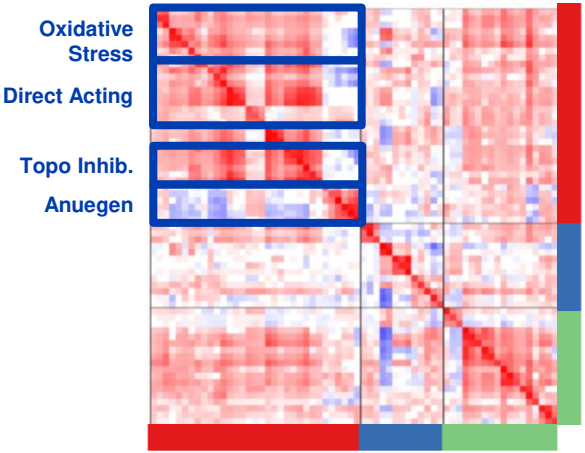


J Lamb et al. Science 2006;313:1929-1935

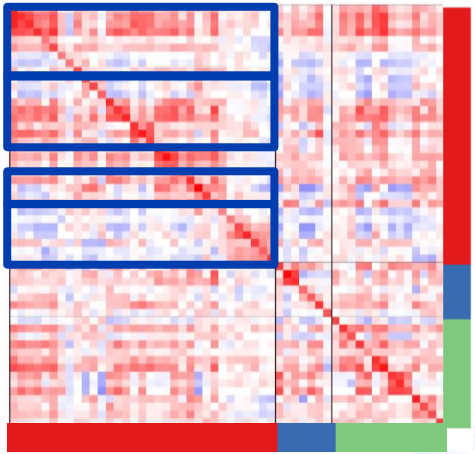


Comparison between chemicals gives a CMap Score between -2 and +2 (based on answers for all genes)

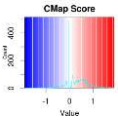
CMap Analysis: Utilization of 3 doses – Affy vs L1000



Affymetrix ~ 40,000 genes



L1000 Expression Profiling ~ 1,000 genes

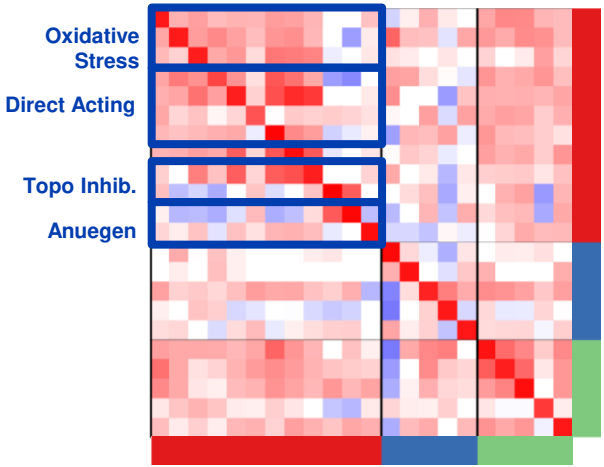


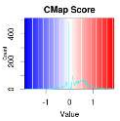
- True Positive (11)
- True Negative (5)
- False Positive (6)

CMap Analysis: Use of one target dose - Impact of platform

Affymetrix ~ 40,000

L1000 Expression Profiling ~ 1,000 genes





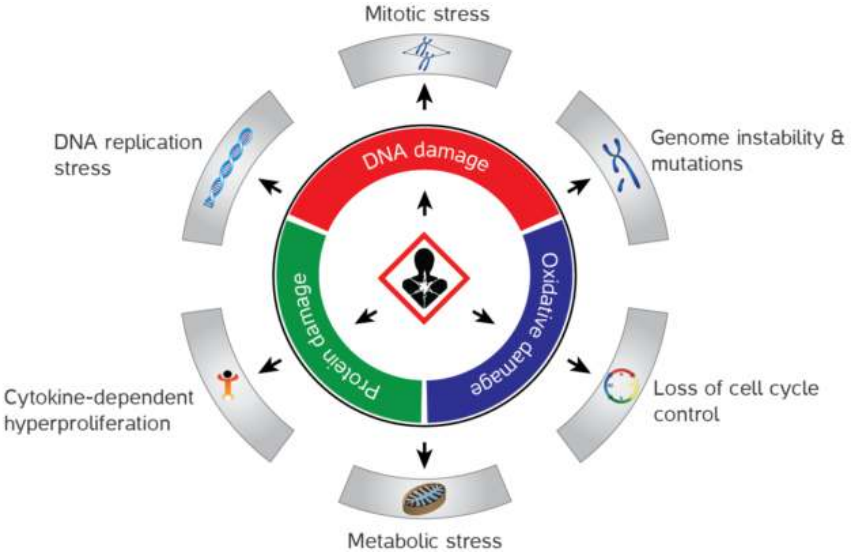
- True Positive (11)
- True Negative (5)
- False Positive (6)

Use of reporter genes – ToxTracker assay

Overview

- **Uses 6 mES GFP reporter cell lines**
- High sensitivity and specificity, according to ToxTracker internal validation
- International validation effort ongoing
- Mechanistic insight into toxicity

Biological damage	Biomarkers
DNA damage	Bscl2, Rtkn
Oxidative stress	Srxn1, Blvrb
Protein damage	Ddit3
Cellular stress	Btg2



Data analysis 3: ToxTracker

Overall results after decoding 22 chemicals

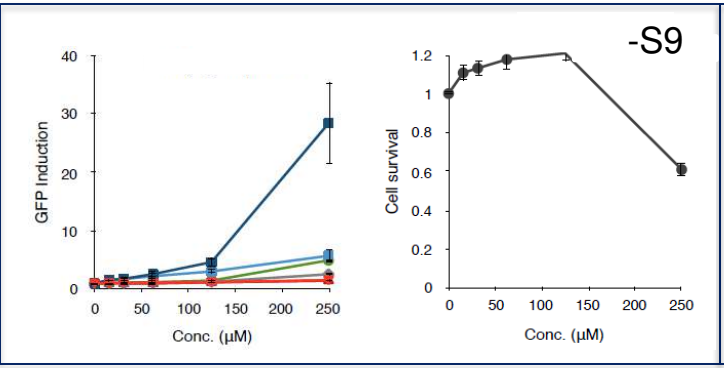
		DNA damage		Oxidative stress		UPR		p53	
		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
	Test compounds								
TN	Cyclohexanone								
TN	Mannitol								
TN	Amitrol								
TN	2-Deoxy-D-glucose								
TN	Sodium diclofenac								
FP	Ethionamide								
FP	Sodium saccharin								
FP	Tertiary-butylhydroquinone								
FP	Curcumin								
FP	Eugenol								
FP	Quercetin								
TP	5-Fluorouracil								
TP	Etoposide								
TP	Vinblastine								
TP	Camptothecin								
TP	Methyl methanesulfonate								
TP	Hydrogen peroxide								
TP	Hydroquinone								
TP	ENU								
TP	O-toluidine								
TP	Colchicine								
TP	Sodium arsenite								
	Controls								
	Cisplatin								
	Diethyl maleate								
	Tunicamycin								
	Aflatoxin B1								

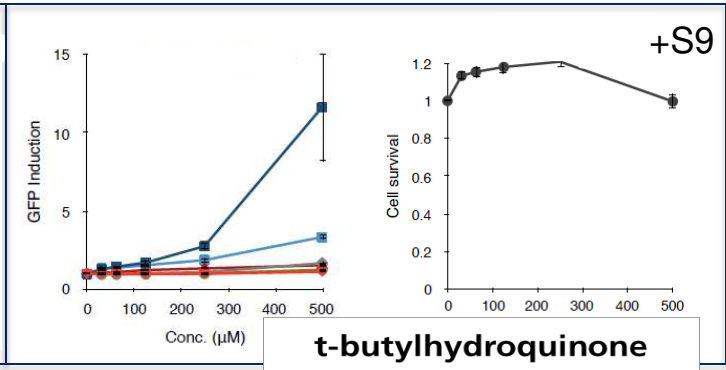
- Positive (>2-fold induction)
- Weak positive (1.5 to 2-fold induction)
- Negative (<1.5-fold induction)
- Inconclusive results

Looking at cutoff by fold increase only (yes/no) misses important information

Data analysis 3: ToxTracker

Example: *tert*-Butylhydroquinone



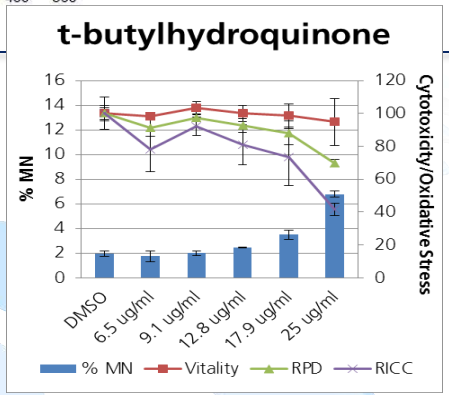


DNA damage
- Bscl2
- Rtkn

Cellular stress (p53)
- Btg2

Oxidative stress
- Srxn1
- Blvrb

Protein damage
- Ddit3



Conclusions part 1

- All three methods show good predictive capacity for set of 22 coded compounds
- C-map and ToxTracker can reveal MoA insights
- C-map shows promise for ‘grouping’ of chemicals since it takes into account toxicological signatures across pathways
- Increasing trend to “condense” (~40,000 genes → 1000) leads to information loss
- Next steps: More in depth analysis of one specific MoA (oxidative stress), added additional chemicals
- Described methods can inform MoA and therefore help risk assessment

Pyrrozolidinly alkaloids

- Pyrrolizidine Alkaloids (PA) are constituents of certain plant families (defense mechanism)
- There are hundreds of PAs but 1,2-unsaturated PAs mainly relevant for safety assessment
- MoA understood/supported well, via in vivo genotoxicity and carcinogenicity data
  - Direct acting mutagen needing metabolic activation
  - Strongly hepatotoxic (poisoning of feedstock, human cases)
- Exposure limits were suggested for PAs in Europe [ECHA 2017 limit: 0.07 mg/kg bw/day]
- Applies for all PA's [sum], but value is driven by the most potent PA
- Relative potencies seem to strongly vary, as a consequence of structural differences [Merz and Schrenk. 2016. Toxicology Letters 263. p44–57]

Can MoA information be used to 'group' PAs, and can 'Key Events' be used to derive relative potency factors (RPF)?

Pyrrozolidinly alkaloids

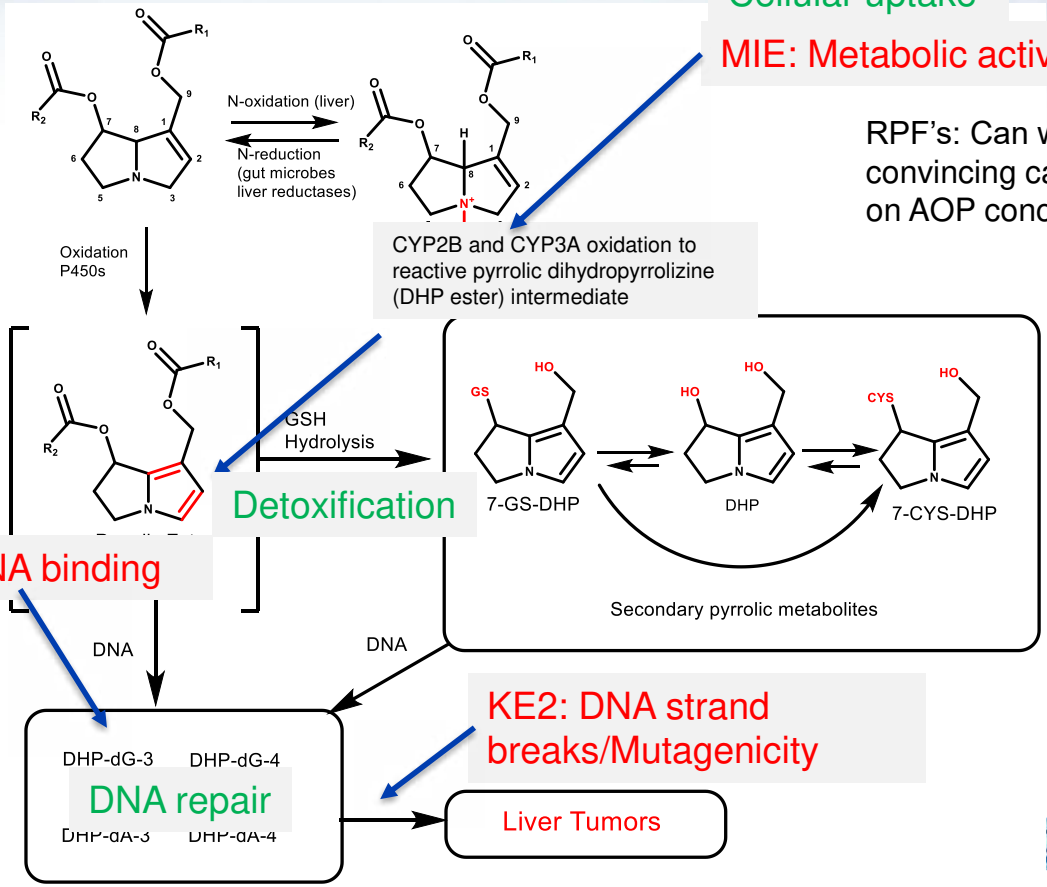
Oral uptake

Gut metabolism

Cellular uptake

MIE: Metabolic activation

RPF's: Can we build a convincing case based on AOP concept?



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Pyrrozolidinly alkaloids

Comprehensive testing program ongoing, aiming to build convincing case based on in vitro and modeling data:

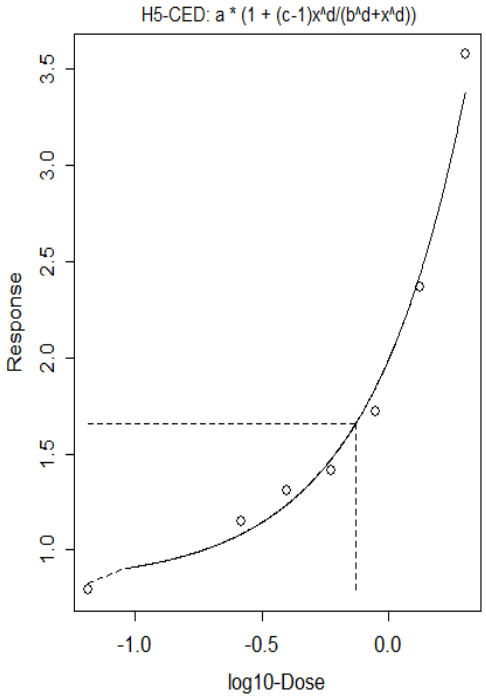
- Cancer potency depends on reactivity of 1,2-unsaturated PAs
  1. Measure rate of reactive metabolite formed (liver microsomes, primary liver cell culture, HepaRG)
  2. Measure their relative genotoxicity potency in metabolically component cell system (micronuclei in HepaRG)
  3. Link 1. and 2. via DNA adduct formation rates.
  4. Consider key additional factors (e.g, oral absorption, fate of 'N-oxides', cellular uptake)
  5. Use 1-4 to calculate RPFs that enable data-driven risk assessment for PAs

Linking expected toxicity via common MoA is prerequisite for acceptance of RPFs

Relative Potency of PAs, initial results

- Developed flow-based micronucleus assay using HepaRG cells (support/training by ILS)
- HepaRG express key enzymes responsible for activation of PAs, e.g, Cyp 3A4
- Investigated 18 PAs to date
- Dose-response curves generated for all (single replicates), aiming to establish optimum dose-range for main studies
- Main studies in triplicates: tight dose-spacing to enable BMD modeling
- Examples:
  - Lasicarpine (suggested RPF 1.0)
  - Lasicarpine N-oxide (RPF ?)
  - Heliotrine (suggested RPF 0.3)
- Critical Effect Size chosen for modeling: 2- fold increase over background

Lasiocarpine, RPF 1.0

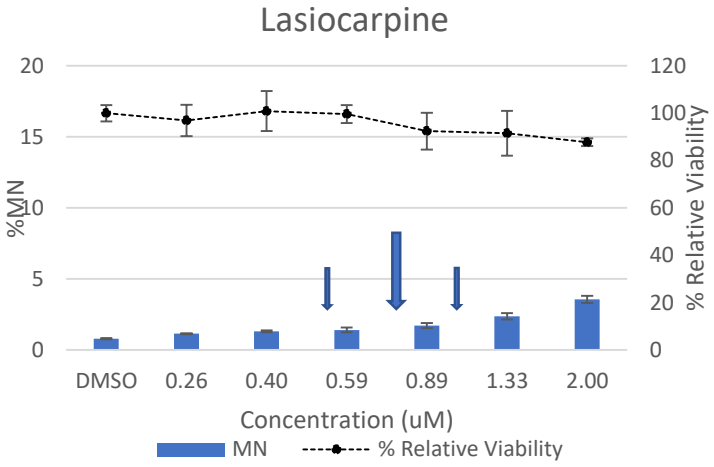


version: 38.9
loglik 10.68
var- 0.00277
a- 0.827
CED- 0.738
c 1000
d- 1.13

b: 329
CES 1
conv : 1
scaling factor on x : 1
dtype : 1

selected : all
removed:
none

CEDL 0.5894
CEDU 0.9051

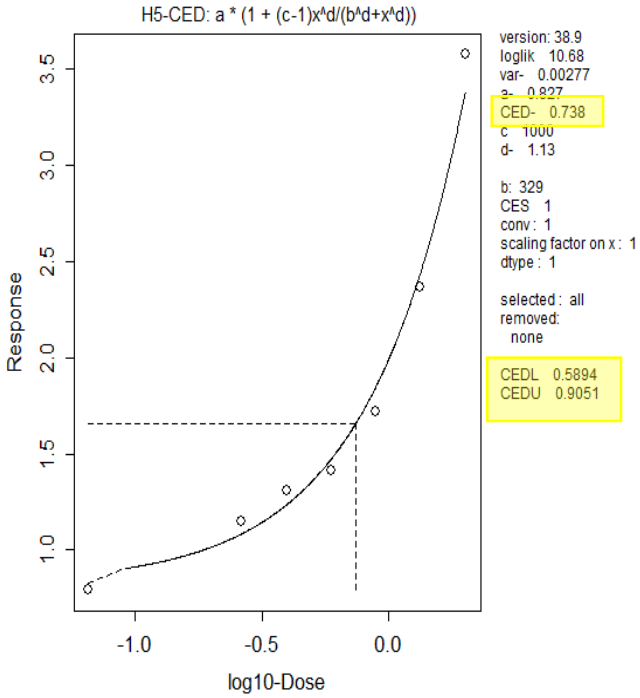


CED = **0.74 uM/ml**

critical effect size = 2-fold increase over control

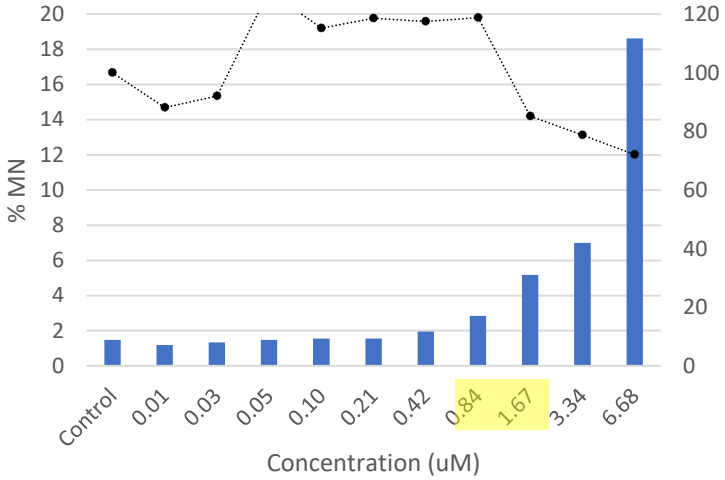
Calculated with PROAST BMD software

Lasiocarpine, RPF 1.0

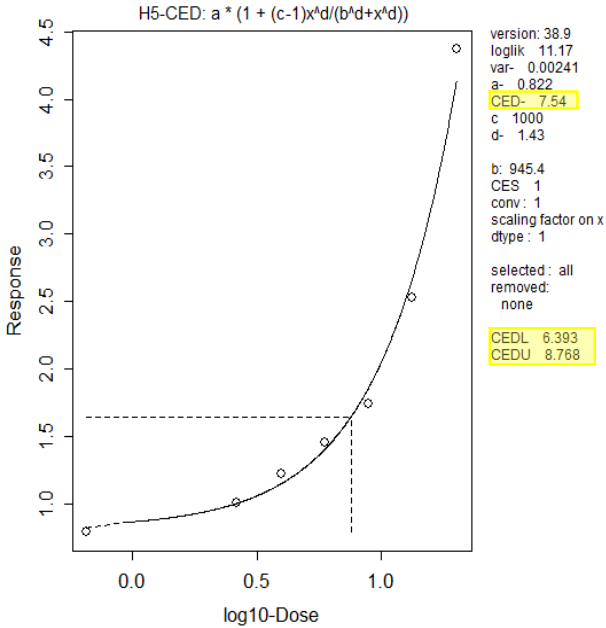


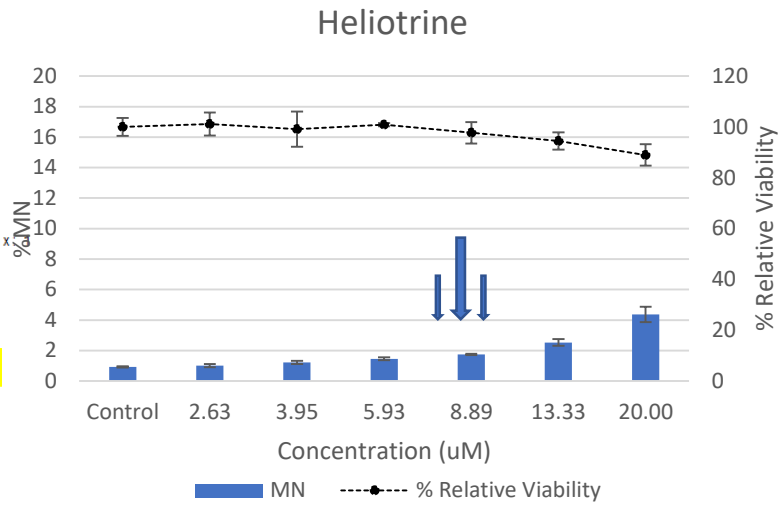
Extremely potent - Dose-finding experiment:

a) Lasiocarpine



Heliotrine, RPF 0.3

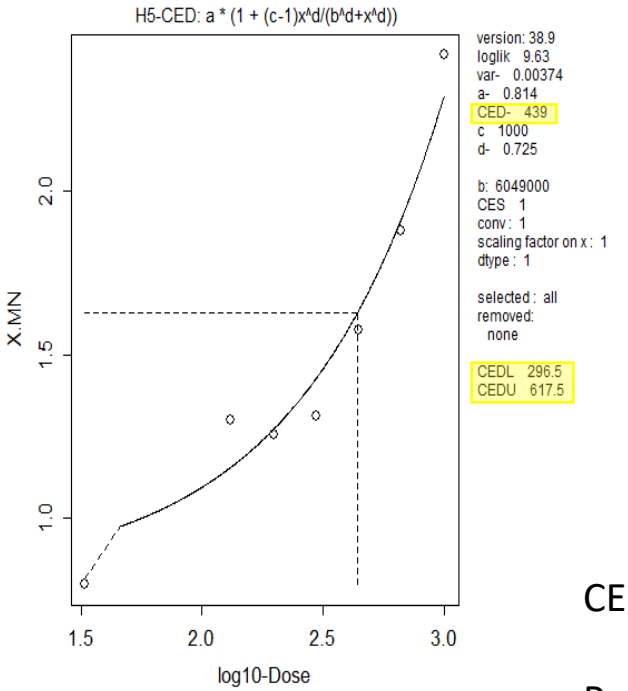


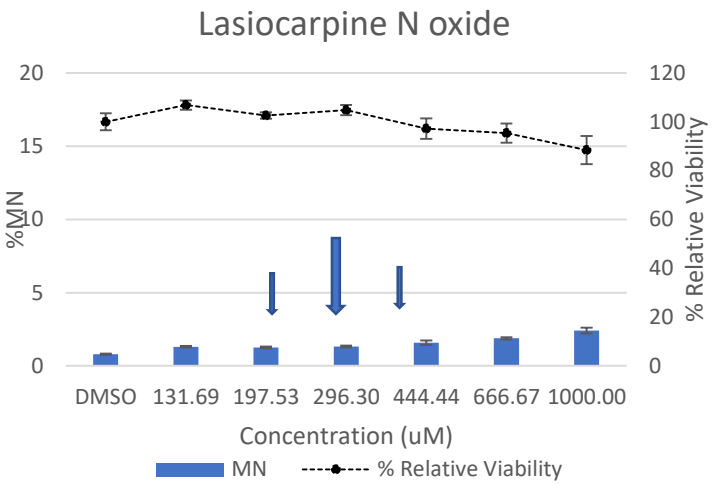


CED = **7.54 uM/ml**

Potency compared to lasiocarpine: **0.1**

Lasiocarpine N Oxide – RPF ?





CED = **439 uM/ml**

Potency compared to lasiocarpine: **0.0016**

Conclusions 2

- Pyrrolizidine Alkaloids (PA) are plant-based impurities with defined genotoxic and carcinogenic MoA
- Current risk management measures define toxicity thresholds that base on assumption all PAs are equipotent
- MIE and KE's can be used to delineate potency
- Additional modulating factors need to be defined per PA for proper calculation of RPF (e.g, oral and cellular uptake, gut metabolism)
- Initial data from micronucleus testing in HepaRG cells show strong differences in potency across PAs
- We believe all parameters can be modeled via *in vitro* and *in silico* data

Open questions

- MOA/AOPs – how high is the burden of proof?
- How can regulatory acceptance be supported?
- How to address ‘uncertainty’ in this context?
- Will the genetox community engage to help drive developments of AOP?
- …..

Acknowledgements

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Health Canada
Andrew Williams
Carol Yauk

Toxys
Giehl Hendrichs
Paula von Rossum

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