Use of Multiparametric Mode of Action Approaches for Genetic Toxicity Assessment: ToxTracker Reporter Cell Lines Versus Gene Array Analysis

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Introduction

In support of the development of a predictive, animal-free genetic toxicology approach, we investigated several different methodologies with varying degrees of complexity. The predictive capacity of these methods was compared by testing 22 chemicals that represent a mix of DNA reactive chemicals, chemicals with known, mixed or unknown modes of action, and chemicals with well-defined in vivo/vitro genotoxicity outcomes. The simplest method, the ToxTracker assay, uses a panel of mouse embryonic stem cell lines, each containing one of six fluorescent reporter genes that detect DNA damage, oxidative stress, cellular stress, or protein damage (Hendriks et al). The two other methods used genomic analysis of data generated with the Affymetrix human genome U219 array platform. For these studies, human lymphoblastoid TK6 cells were treated with the chemicals and aliquots of the cell suspensions were set aside for genomic analysis. The remaining suspension was used to measure micronuclei (MN), cell viability and intracellular thiol levels in parallel. These results then became the basis for the samples selected for genomic analysis. The gene array data were then analyzed using two different approaches, the 65-gene method (TGx28.65) developed by a HESI expert team (Li et al) and an in-house method utilizing connectivity mapping (CMap; Lamb et al).

Methods and Methods

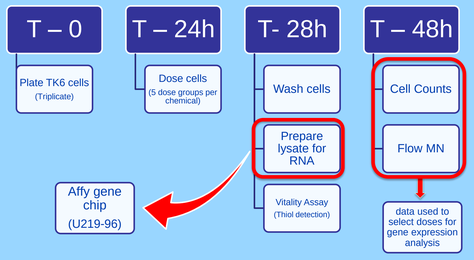
Gene expression studies: cells, chemical treatments and parameters measured

TK6 human lymphoblastoid cells were maintained at or below 1x10^6 cells/ml in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and 1% penicillin- streptomycin. Cells were seeded at 50,000 cells/ml for 24 hours before administration of test chemicals at doses up to 10 mM. After 4 hours, aliquots of the cells were removed for RNA isolation for gene expression analysis using the Affymetrix platform and vitality estimation by intracellular thiol measurement. The remaining cells were resuspended in fresh media and incubated for an additional 20 hours before measurement of cell counts and micronuclei using the Litron In Vitro MicroFlow procedure including addition of latex counting beads to obtain a relative viability measure. Samples were analyzed using a BC FACS Canto II and were analyzed using FACS Diva v6.

Selected Chemicals Tested (balanced set)

True Negative	False Positive	True Positive	
Cyclohexanone	Curcumin	ENU	Hydrogen Peroxide
D-Mannitol	Tert-butyl hydroquinone	MMS	O-toluidine
Amitrol	Ethionamide	Sodium Arsenite	5-Fluorouracil
Sodium Diclofenac	Sodium Saccharin	Camptothecin	Etoposide
2-deoxy-D-glucose	Eugenol	Vinblastine	Colchicine
	Quercetin		Hydroquinone

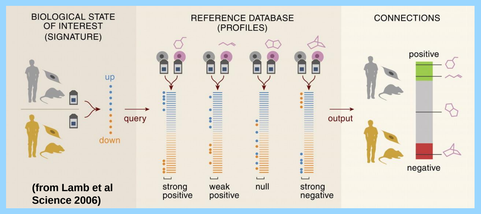
Gene Expression Studies Method Overview



TGx-28.65

- 65-gene signature data generated using probability analysis, PCA, cluster analysis
- Based on microarray analysis
- Identifies DNA damage inducing (DDI) agents (not anuegens)
- Data analysis performed by Health Canada (Carole Yauk, Andrew Williams)

The Connectivity Mapping (CMap) Concept



Toxys ToxTracker® Assay

Panel of six different mouse ES-GFP reporter cell lines representing four distinct biological responses associated with carcinogenesis, i.e. general cellular stress, DNA damage, oxidative stress, and the unfolded protein response. Cells were seeded at 50,000 cells/well for 24 hours before administration of the test chemicals at doses up to 1 mM in the presence or absence of rat S9 liver extract. For metabolic activation studies, the cells were resuspended in fresh medium after 3 hours and incubated for an additional 21 hours. After 24 hours, cell counts and induction of the various GFP reporters were determined using a Guava easyCyte 8HT flow cytometer.

ToxTracker assay overview

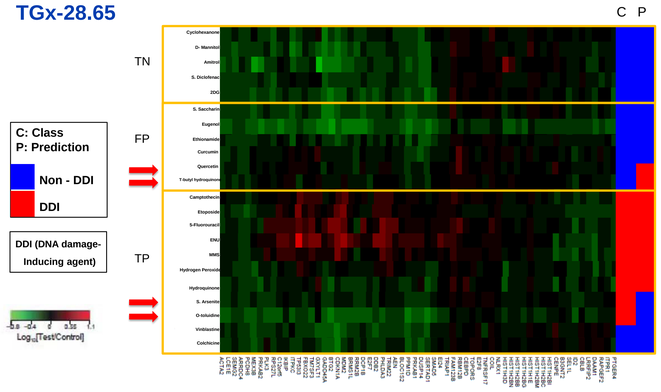
- Early *in vitro* toxicity screening for lead selection and optimization
- Rapid and cost effective
- High sensitivity and specificity
- Mechanistic insight into toxicity

six mouse ES-GFP reporter cell lines

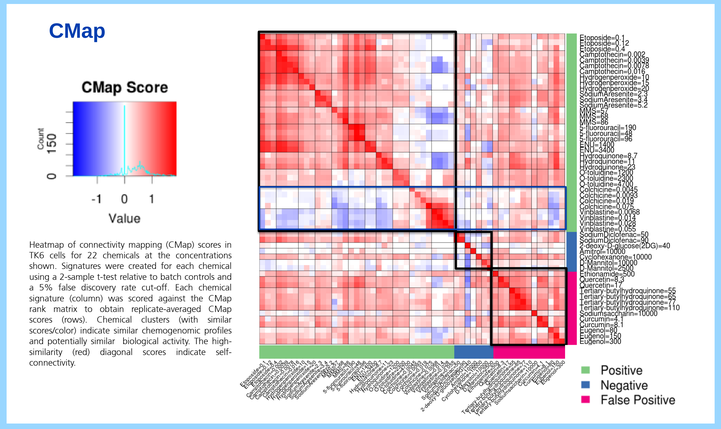
Biological damage	Cellular pathway	Biomarker gene
DNA damage	ATR/Chk1 DNA damage signaling	Bscl2
	NF-kB signaling	Rtkn
Oxidative stress	Nrf2 antioxidant response	Srxn1
	Nrf2-independent	Blvrb
Protein damage	Unfolded protein response	Ddit3
Cellular stress	p53 signaling	Btg2

Results

TGx-28.65

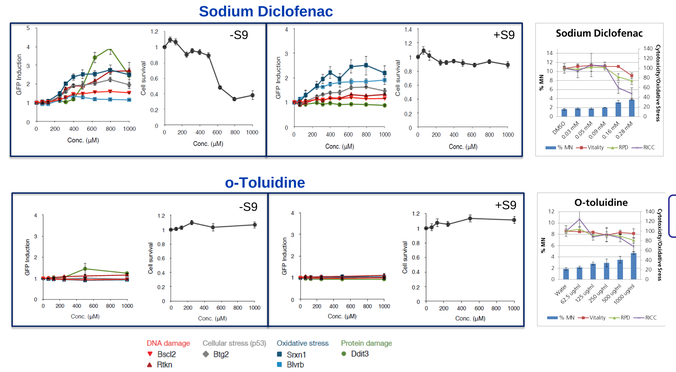


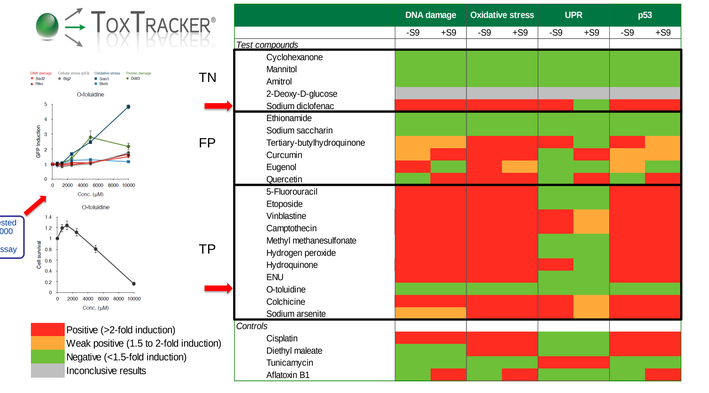
CMap



Heatmap of connectivity mapping (CMap) scores in TK6 cells for 22 chemicals at the concentrations shown. Signatures were created for each chemical using a 2-sample t-test relative to batch controls and a 5% false discovery rate cut-off. Each chemical signature (column) was scored against the CMap rank matrix to obtain replicate-averaged CMap scores (rows). Chemical clusters (with similar scores/color) indicate similar chemogenomic profiles and potentially similar biological activity. The high-similarity (red) diagonal scores indicate self-connectivity.

ToxTracker





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Summary and Conclusions

- **Our goal was to compare different methodologies using genomic biomarkers that have been developed for classifying chemicals by their MoA and allow us to draw conclusions regarding primary/secondary genotoxicity.**
- **Reasonable alignment of predictive capacity was seen from all methods (overall predictivity was >80%) and nearly all discrepancies among assays could be explained by the top concentration tested or the dose selected for analysis.**
- **Increasing trend to "condense" (~40,000 genes→65→6), but at what cost? Untapped data may be beneficial for read-across, e.g. CMap database. Further analysis is ongoing.**
- **Overall, these results support the utility of multiparametric approaches for development of signatures representing various MoA's from key chemical classes and may allow connection to data rich chemicals for read across.**
- **Ultimately, we hope to incorporate this approach into a predictive genotoxicity risk assessment strategy.**

References

- **Hendriks et al., 2016. The extended ToxTracker assay discriminates between induction of DNA damage, oxidative stress, and protein misfolding. Toxicological Sciences 150: 190-203.**
- **Li et al., 2015. Development of a toxicogenomics signature for genotoxicity using a dose-optimization and informatics strategy in human cells. Environmental and Molecular Mutagenesis 56:505-519.**
- **Lamb et. al., 2006. The connectivity map: Using gene-expression signatures to connect small molecules, genes, and disease. Science 313: 1929-1935.**