Quantitative Nuclease Protection Assays (qNPA™) as Windows into Chemical-Induced Adaptive Response in Cultures of Primary Human Hepatocytes (Concentration- & Time-Response)

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Abstract

Cultures of primary human hepatocytes have been shown to be dynamic *in vitro* model systems that retain liver-like functionality (e.g. metabolism, transport, induction). We have utilized these culture models to interrogate 309 ToxCast chemicals. The study design characterized both concentration- and time-response effects of these chemicals across two preparations of human hepatocytes by mRNA expression, CYP1A enzymatic activity (EROD), and cell morphology. mRNA expression was determined using quantitative nuclease protection assays (qNPA™) with the Omix™ Imaging System (HTG, Tucson, AZ). Fourteen liver-related human gene targets ABCB1, ABCB11, ABCG2, SLCO1B1, CYP1A1, CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP3A4, UGT1A1, GSTA2, SULT2A1, HMGCS2 (Figure 1) were monitored based on their role in liver xenobiotic metabolism, hepatic transport, and sensitivity to receptor pathways (AhR, CAR, PXR, PPARα, FXR). These data were analyzed relative to negative and positive control receptor activators. These data were fit to sigmoidal concentration-response model (Hill equation) to generate important potency and efficacy parameters (e.g EC_{50}, E_{max}, Hillslope, R^2 etc…). Concordance analysis was performed on the internal replicate ToxCast chemicals to assess the reproducibility of the assays.

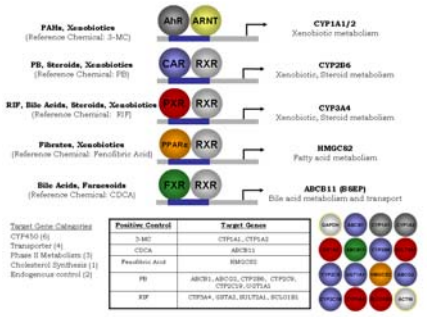
In addition, techniques from machine learning were leveraged to cluster compounds having similar gene response profiles. The concentration-response of a compound was abstracted as a vector (rather than classical scalar representations associated with standard microarray analysis) and used in algorithms such as K-means and algometric clustering, as well as creating representative phylogenies. Unique to this approach is the ability to assess if compounds behave similarly in a temporal sense. Using this methodology we were able to correlate how a chemical's behavior compares with other compounds through time, as well as correlating gene targets with one another. These chemical signatures were further correlated with in vivo endpoints in relative risk assessments to define *in vitro* profiles that appear to be related to phenotypic outcomes.

Methods

Primary cultures of human hepatocytes were prepared from human liver tissue derived from two separate male donors (Hu776 and Hu778). Hepatocyte cultures were treated daily for two consecutive days with fresh dosing solutions containing appropriate concentrations of the 320 ToxCast chemicals, vehicle control (0.2% DMSO) and positive control inducers. Cultures for each treatment group (i.e. media, vehicle (0.2% DMSO), positive control inducers (multiple concentrations), and the ToxCast chemicals (multiple concentrations) were observed and cell morphology was assessed relative to vehicle control cultures at each harvest time point (0, 6, 24, and 48 hours). mRNA content was assessed at each harvest point using a quantitative Nuclease Protection Assay (qNPA™).

The raw plate data were annotated with matching chemical and dosage information and compiled in a database. Concentration-response curves were fit using the R statistical language modeled by the Hill-equation. Gene reference chemical selection was based on each positive control's computed Z-Factor. Gene to gene correlations were calculated using Pearson's correlation and computed using the observed dynamic range for each gene. The entire ToxCast library was then clustered using all data points from a compound's response. Concordance was also calculated to assess assay variability.

Figure 1: Gene Array and Assay Design



An array of 14 hepatic related sentinel gene targets (and 2 endogenous controls) for 5 receptor pathways to probe chemical-biological interactions in cultures of primary human hepatocytes with quantitative nuclease protection assays (qNPA™).

Results

Figure 2: Positive Control Curves

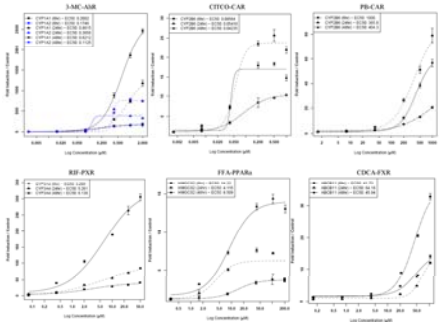


Figure 3: Select Chemical Distributions

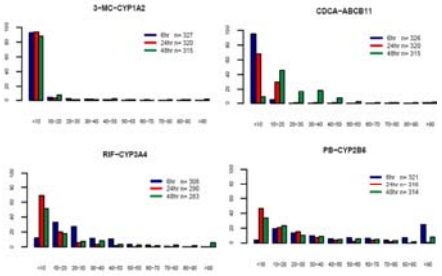


Figure 4: Representative ToxCast Chemicals

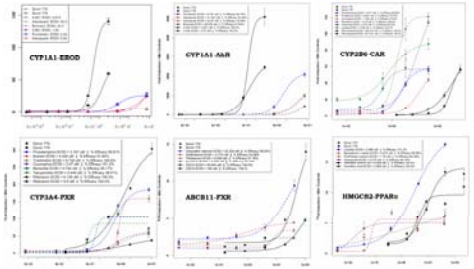


Figure 2: The positive control responses for each respective gene target across all three time points (6,24,48hrs).

Figure 3: Maximal efficacy histograms for CYP1A2, CYP2B6, CYP3A4, and ABCB11 measured against the respective positive control for the entire ToxCast chemical library.

Figure 4: Compounds comprising the most potent and efficacious ToxCast chemicals and the respective gene's reference chemical.

Conclusions and Discussion

We have characterized the bioactivity of the 309 unique chemicals currently in the ToxCast library in cultures of primary human hepatocytes over ranges of concentration and time. Correlations were observed between activation of key receptor pathways and certain rodent *in vivo* toxicity endpoints. These correlations indicate the value of using this *in vitro* hepatocyte culture systems in predictive toxicity modeling, and identifies putative human toxicity pathways for specific disease endpoints.

Figure 5: In Vivo Relative Risk

Rodent *In Vivo* Relative Risk

Gene	Endpoint	Relative Risk
ABCB11	Liver Apoptosis Necrosis**	1.63*
CYP1A1	Liver Apoptosis Necrosis	1.19*
CYP1A2	Liver Apoptosis Necrosis	1.71*
CYP2B6	Liver Hypertrophy	2.20
CYP2B6	Thyroid Tumors	2.03
CYP3A4	Liver Tumors	2.35
HMGCS2	Liver Tumors	2.60
SULTA1	Thyroid Hyperplasia	2.26

Relative Risk of gene expression after 48-hour exposure (24-hour RR observations ≥ 2 noted with asterisk*) to predict the in vivo chronic toxicity of ToxCast™ chemicals. Five genes represent chemical effects on CAR (CYP2B6), FXR (ABCB11), PPARα (HMGCS2), AhR (CYP1A1), and PXR (CYP3A4) receptor pathways. All endpoints are Rat except for ABCB11Mouse Liver Apoptosis Necrosis which is noted by **

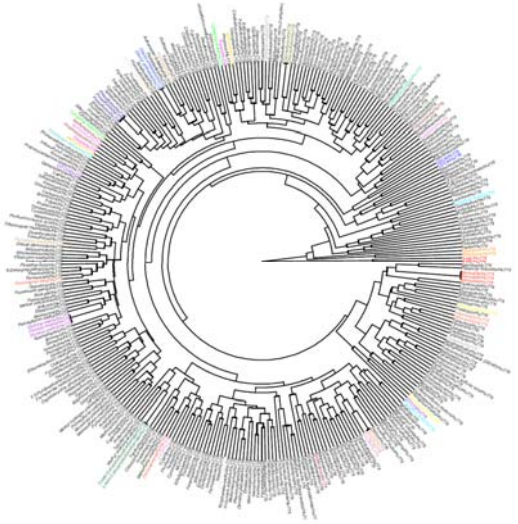
Figure 6: Gene Correlations

Notable Gene Correlations

Gene 1 (time)	r	Gene 2 (time)
CYP1A1 (24)	.9445	CYP1A2 (24)
ABCB11 (48)	.9053	SLCO1B1 (48)
ABCB11 (48)	.8859	ABCG2 (48)
ABCG2 (48)	.8645	SLCO1B1 (48)
CYP1A1 (24)	.8610	CYP1A2 (48)
ABCB1 (48)	.8515	ABCG2 (48)
CYP1A1 (6)	.8312	CYP1A2 (24)
CYP1A2 (24)	.8220	CYP1A2 (48)
ABCB1 (48)	.6156	CYP3A4 (48)
CYP2C9 (24)	-.2103	HMGCS2 (6)

Pearson correlation between all 14 gene targets across 3 time points. The statistic used in calculating the correlation was the dynamic range ($E_{max} - E_{min}$) of a gene's response. The above table contains notable correlations.

Figure 6: Clustering and Dendrograms



A dendrogram of the ToxCast™ 320 chemical library created using the vector abstraction of each compound's dose-response for all 14 gene targets across all three time points. Different donors were Z-scored independently, to reduce both inter-donor variability and bias towards more efficacious genes. Incorporating all three time points into a 210 dimensional space (70 dimensions per time point x 3 time points = 210) resulted in more accurate clustering of EPA replicates that were blinded during the study as well as the positive controls. Z-scoring donors independently also drastically reduced donor dependence; without it the clustering results were greatly affected by the donor in which the compound was tested. Corresponding replicates and positive controls are given the same color.

Although this work was reviewed by EPA and approved for publication, it may not necessarily reflect official Agency policy.

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