

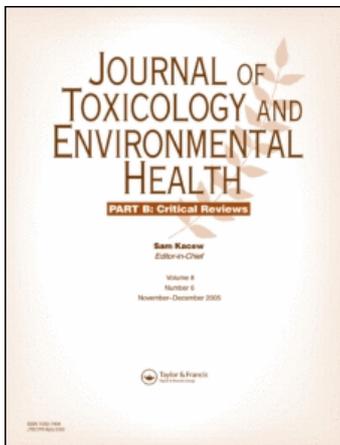
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XENOBIOTIC-METABOLIZING ENZYME AND TRANSPORTER GENE EXPRESSION IN PRIMARY CULTURES OF HUMAN HEPATOCTES MODULATED BY TOXCAST CHEMICALS

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Primary human hepatocyte cultures are useful *in vitro* model systems of human liver because when cultured under appropriate conditions the hepatocytes retain liver-like functionality such as metabolism, transport, and cell signaling. This model system was used to characterize the concentration- and time-response of the 320 ToxCast chemicals for changes in expression of genes regulated by nuclear receptors. Fourteen gene targets were monitored in quantitative nuclease protection assays: six representative cytochromes P-450, four hepatic transporters, three Phase II conjugating enzymes, and one endogenous metabolism gene involved in cholesterol synthesis. These gene targets are sentinels of five major signaling pathways: AhR, CAR, PXR, FXR, and PPAR α . Besides gene expression, the relative potency and efficacy for these chemicals to modulate cellular health and enzymatic activity were assessed. Results demonstrated that the culture system was an effective model of chemical-induced responses by prototypical inducers such as phenobarbital and rifampicin. Gene expression results identified various ToxCast chemicals that were potent or efficacious inducers of one or more of the 14 genes, and by inference the 5 nuclear receptor signaling pathways. Significant relative risk associations with rodent *in vivo* chronic toxicity effects are reported for the five major receptor pathways. These gene expression data are being incorporated into the larger ToxCast predictive modeling effort.

The U.S. Environmental Protection Agency (EPA) ToxCast research project is evaluating a substantial collection of *in vitro* screening assays for the ability to profile the bioactivity of environmental compounds and generate data predictive of *in vivo* toxicity. Assessing the toxicity of environmental compounds presents a

significant challenge to the producers, users, regulators, and other stakeholders of pesticides, industrial chemicals, nanomaterials, and other environmental contaminants. The current approach to assessing potential human toxicity of environmental contaminants is primarily a series of *in vivo* studies in rodents and

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other species. This approach can be slow and expensive, and has various uncertainties associated with predicting hazards relevant to humans. In order to (1) increase the absolute number of compounds tested, (2) reduce the amount of animal testing, (3) lower the high cost of *in vivo* testing, and (4) provide better mechanistic understanding of human toxicity, there is increasing pressure to create alternatives to the current testing paradigm with more sophisticated batteries of *in vitro* assays. The primary goal of the ToxCast program is to screen a large number of industrial chemicals, pesticides, and other environmental compounds and provide toxicity predictions useful for chemical prioritization (Dix et al., 2007) on thousands of compounds (Judson et al., 2009). The first phase of ToxCast is applying approximately 600 *in vitro* assays to a set of 320 chemicals, most of which are pesticide active ingredients for which detailed *in vivo* toxicity studies have been carried out and the results curated into the ToxRefDB database (Martin et al., 2009). The National Research Council (NRC) recently laid out a vision that essentially endorses efforts like these having potential to transform toxicity testing from the current *in vivo*-focused paradigm to one in which *in vitro* assays provide mechanistic data on human toxicity pathways (NRC, 2007). Such a shift will require a wide range of assays probing different toxicity pathways involved in cancer, developmental and reproductive toxicity, and neurotoxicity. Data presented herein originate from an assay designed to monitor receptor pathway perturbations resulting from exposure to the ToxCast chemicals in metabolically competent cultures of primary human hepatocytes.

Cultures of primary human hepatocytes are considered highly representative *in vitro* model systems for the human liver (LeCluyse, 2001). Current Food and Drug Administration (FDA) Guidance for Industry (2006) states that cultures of primary human hepatocytes are the most reliable *in vitro* model system to evaluate the induction potential of new molecular entities (FDA, 2006). Sandwich cultured hepatocytes, maintained between a layer of substratum

(e.g., type I collagen) and overlay of extracellular matrix, represent a viable three-dimensional environment that can maintain functional hepatocytes for multiple days/weeks to study complex processes such as chemical-induced changes in hepatic gene expression (LeCluyse et al., 1999; LeCluyse, 2001).

Nuclear receptor-mediated regulation of gene expression represents an important hepatic response to exposure to both endogenous and exogenous substrates (Nakata et al., 2006). These receptors regulate multiple gene targets involved in absorption, metabolism, disposition, and excretion of endogenous and foreign chemicals (and metabolites). For the liver, a list of the most relevant nuclear hormone receptors includes pregnane X receptor (PXR, NR1I2), constitutive androstane receptor (CAR, NR1I3), peroxisome proliferator activated receptor alpha (PPAR α , NR1C1), and farnesoid X receptor (FXR, NR1H4). In addition, the aryl hydrocarbon receptor (AhR), which is not a member of the nuclear receptor superfamily, is also important for CYP1A induction.

In the present study an array of 14 relevant hepatic gene targets and 2 endogenous controls was designed to probe chemical-biological interactions in cultures of primary human hepatocytes with quantitative nuclease protection assays (qNPA) (Roberts et al., 2007). These target genes serve as sentinels for key nuclear receptor pathways: CYP1A1/2 for AhR, CYP2B6 for CAR, CYP3A4 for PXR, HMGCS2 for PPAR α , and ABCB11 for FXR. Using this array with cell morphology observations, the ToxCast chemicals were characterized over five concentrations and four time points and compared to prototypical receptor activators that served as reference chemicals. Gene expression data were fitted to concentration-response curves to assess the relative potencies, efficacies, profiles, and trends across concentration and time. The ability to predict *in vivo* hepatotoxicity with these *in vitro* data was demonstrated for a limited number of endpoints in rodent liver. These data are being incorporated into the larger ToxCast predictive modeling effort (<http://www.epa.gov/ncct/toxcast>). This study demonstrated the utility of

assessing concentration- and time-response profiles of relevant nuclear receptor target genes in cultures of primary human hepatocytes as a means to characterize their chemical potency (concentration at which a biological response is observed) and efficacy (magnitude of a response observed above negative control values) in modulating biological and toxicological pathways.

MATERIALS AND METHODS

ToxCast Chemical Library

Phase I of the U.S. EPA ToxCast program employs a chemical library containing 320 compounds with appropriate physicochemical properties to ensure compatibility with a wide range of cell-free and *in vitro* cellular assays (<http://www.epa.gov/ncct/toxcast>). The ToxCast320 chemical library consists of 309 unique chemical structures meeting physicochemical property requirements for high-throughput screening. Five substances were tested in duplicate (separately sourced) and three chemicals were tested in triplicate (sample replicates) for internal quality control purposes. Most of the compounds are pesticide active ingredients associated with extensive *in vivo* toxicity data generated in support of their registration process with the U.S. EPA. These data have been extracted from documents, standardized, and compiled in the U.S. EPA ToxRefDB relational database (supplemental file 2, <http://www.epa.gov/NCCT/toxrefdb>). Mammalian *in vivo* chronic toxicity endpoints at the time of this analysis were available in ToxRefDB for 246 (rat) and 235 (mouse) of the 309 unique ToxCast chemicals. Chronic toxicity data was available for 190 chemicals in both species. The full list of chemicals is available with quality reviewed structure-annotation from the U.S. EPA DSSTox website (http://www.epa.gov/ncct/dsstox/sdf_toxcst.html). Chemical samples were procured and plated by BioFocus DPI (San Diego, CA; U.S. EPA contract EP-D-07-060). Supplier-provided certificates of analysis indicated purity >97% for the large majority of chemicals (87%), and >90% purity for all but a few instances of technical grade or

known mixtures. Follow-up analysis of an original solution plate by BioFocus DPI using liquid chromatography (LC) and gas chromatography/mass spectrometry (GC/MS), subsequent to assay screening confirmed mass identification, stability, and purity in excess of 90% for over 87% of the chemical library, with follow-up analysis underway for the remaining compounds. Summary quality control (QC) information mapped to chemical sample and solution IDs will be provided on the ToxCast website as an auxiliary chemical file (<http://www.epa.gov/ncct/toxcast>). Compounds were dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 20 mM. For testing in concentration-response format, serial dilutions were performed in DMSO followed by aqueous dilution in cell culture medium. In order to avoid bias, chemical names were blinded for testing at CellzDirect. The chemical samples (20 mM stocks in DMSO) were provided by the U.S. EPA in four 96-well plates and stored at approximately -80°C prior to use. The stock solutions were diluted daily in culture medium such that the final DMSO concentration was 0.2% to achieve the final dosing concentrations of 0.004, 0.04, 0.4, 4 or 40 μM .

Reference Chemicals and Other Reagents

Phosphate-buffered saline was purchased from Invitrogen (Gibco, Grand Island, NY). ITS+ was purchased from BD Biosciences (San Jose, CA). Williams E medium, Hanks balanced salt solution (HBSS), dexamethasone (DEX), DMSO, 3-methylcholanthrene (3-MC), phenobarbital (PB), rifampicin (RIF), fenofibric acid (FFA), 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO), and chenodeoxycholic acid (CDCA) were obtained from Sigma Chemical Co. (St. Louis, MO). DMEM/Ham's F-12 medium (Cellgro) was purchased from Mediatech (Manassas, VA). Stock solutions were prepared and stored at 4°C or -20°C . DMSO, water, and other solvents were high-performance liquid chromatography (HPLC) grade or the highest quality available. High Throughput Genomics (HTG, Tucson, AZ)

reagents and methodologies were used for the nuclease protection assays.

Isolation of Hepatocytes and Donor Information

Primary cultures of human hepatocytes were prepared from human liver tissue derived from two separate male donors (Hu776 and Hu778) and had initial viabilities of 91 and 95%, respectively, at the time of plating. Donor Hu776 was a male Caucasian, 41 yr of age, weighing 180 lb, standing 5'10" tall, who consumed 4–6 alcoholic beverages per week and occasionally chewed tobacco. Donor Hu778 was a male Caucasian, 55 yr of age, weighing 162 lb, standing 5'6" tall, with no history of alcohol or tobacco consumption. Tissue specimens used for these studies were derived from the normal margins of resected liver tissue that was resected due to the presence of metastatic colon tumors. The research was carried out in accordance with the principles of the current version of the Helsinki Declaration. Each patient whose tissue was used in this study was fully consented under an institutional review board (IRB) application approved by the individual institutions from patients undergoing liver resection surgery. Eligible patients were between the ages of 18 and 75 yr and were not restricted to any gender or ethnic grouping. All samples were collected and preserved at the participating institution and shipped to CellzDirect's facility in Durham, NC, for processing under protocols approved during the IRB application process.

Hepatocytes were isolated by a modification of the two-step collagenase perfusion method described previously (LeCluyse et al., 2005). Final cell viability, prior to plating, was determined by the trypan blue exclusion test and was $\geq 90\%$ in both preparations. Following isolation, hepatocytes were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum, insulin (4 $\mu\text{g}/\text{ml}$), and DEX (1 μM) and added to 96-well plates (BioCoat, BD Biosciences, San Jose, CA) coated with a simple collagen, type I, substratum. Hepatocytes were allowed to attach for 4–6 h at 37°C in a humidified culture chamber

with 95% relative humidity/5% air/CO₂. After attachment, culture vessels were swirled and medium containing debris and unattached cells was aspirated. Fresh ice-cold serum-free DMEM/Ham's F12 containing 50 nM DEX, 6.25 $\mu\text{g}/\text{ml}$ insulin, 6.25 $\mu\text{g}/\text{ml}$ transferrin, 6.25 ng/ml selenium (ITS+), and 0.25 mg/ml ECM was added to the culture vessels and immediately returned to the culture chamber. Medium was changed on a daily basis thereafter. Cultures of hepatocytes were maintained for 24–48 h prior to initiating experiments with the ToxCast chemicals.

Treatment of Hepatocytes With Chemicals

Human hepatocyte cultures were treated daily for two consecutive days with fresh dosing solutions containing appropriate concentrations of the 320 ToxCast chemicals (chemical identities were blinded to CellzDirect and other ToxCast collaborators), vehicle control (0.2% DMSO), and positive control inducers as summarized in Table 1. Due to the large number of treatment groups and chemicals examined, the ToxCast chemicals were divided into two groups (spanning two independent cultures of hepatocytes from separate donor preparations (Hu778: ToxCast plates 1 and 2; Hu776: ToxCast plates 3 and 4). Each preparation of human hepatocyte cultures was treated with respective vehicle control, medium only control, set of six positive controls/reference chemicals for the five hepatic receptor pathways, and a subset of eight of the ToxCast chemicals (indoxacarb, pyriithiobac-sodium,

TABLE 1. Treatment Summary for Reference Chemicals and ToxCast Chemicals

Treatment	Concentrations
Media	
Negative control	0.2% DMSO
Positive control: 3-MC	2, 1, 0.4, 0.08, 0.016, 0.0032 μM
Positive control: PB	1000, 500, 200, 40, 8, 1.6 μM
Positive control: CITCO	200, 100, 40, 8, 1.6, 0.32 nM
Positive control: RIF	50, 25, 10, 2, 0.4, 0.08 μM
Positive control: FFA	200, 100, 40, 8, 1.6, 0.32 μM
Positive control: CDCA	100, 50, 20, 4, 0.8, 0.16 μM
ToxCast chemicals:	40, 4, 0.4, 0.04, 0.004 μM

norflurazon, cyhalofop-butyl, methomyl, thiram, acetochlor, and propiconazole). These replicate data, coupled with internal replicates designed within the blinded ToxCast chemical library, provided additional data to evaluate interindividual differences between hepatocyte preparations and their potential impact on chemical profiles.

Cell Morphology Assessment

Cell morphology and integrity were evaluated using phase-contrast microscopy as an indicator of hepatocyte cell health (Tyson & Green, 1987). 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, or 48 h). Any discernable morphological alterations such as changes in cell shape, nucleus size/shape, cytoplasmic alterations, and accumulation of vacuoles suggestive of dilated organelles and lipid droplets (Guillouzo et al., 1997) that were observed as a consequence of chemical exposure were recorded in images captured using a Zeiss Axiovert inverted research microscope equipped with phase-contrast optics, a 3 CCD camera, and a computer with image capture and analysis software. Images were evaluated at the conclusion of the study to assess changes in cell integrity and account for the effects of chemical exposure on uncharacteristic changes in concentration-dependent gene expression profiles. The results from these determinations of apparent cytotoxicity were annotated (yes/no) alongside the corresponding qNPA data for reference.

Nuclease Protection Assays (qNPA)

At the conclusion of each treatment period, hepatocyte cultures (96-well) were washed with 1 volume of HBSS, lysed by addition of 25 μ l ArrayPlate lysis buffer (HTG, Tucson, AZ), and 70 μ l/well of Denaturation Oil, denatured by incubation at 95°C for 10 min, and frozen at approximately -70°C until

analysis by nuclease protection assay (qNPA) (Roberts et al., 2007).

For qNPA analysis, cell lysates were thawed at 50°C for approximately 30 min, qNPA probes were added, and samples were incubated at 95°C for 10 min to begin the detection process by denaturing the target RNA, dissociating the duplexes and secondary structure hybridization. At the conclusion of the hybridization period, S1 nuclease reagent was added to each sample to digest all non-protected nucleotides at 50°C for 60–90 min. At the conclusion of the S1 nuclease digestions, all reactions were stopped by transfer of all the samples to fresh plates containing stop solution and incubated at 95°C for 15 min to deactivate the enzyme, dissociate the mRNA/DNA probe heterodimers, and hydrolyze the resulting single stranded mRNA, leaving a stoichiometric amount of single-stranded DNA nuclease protection probe, unmodified in sequence, as the only intact oligonucleotide left in the sample. Neutralization solution was subsequently applied to cooled (room temperature) plates, and samples were transferred to ArrayPlates for overnight incubations at 50°C to allow probes to be captured onto programmed locations on the ArrayPlates. Half of the nucleotides comprising each nuclease protection probe are utilized for capture hybridization to the array.

At the completion of the array capture of probes, plates were washed, detection linkers were hybridized to the other half of each nuclease protection probe, plates were washed again, and detection enzymes were applied. The final step in the process was the imaging of the plates with the OMIX Imaging System (HTG, Tucson AZ). The quantity of protected nuclease protection probe, and hence target mRNA in each well, was proportional to the luminescence intensity of the labeled detection oligonucleotides that bind each of the 16 spots within each well of a 96-well plate. Luminescence data were generated using the OMIX Imaging System software to generate endogenous control normalized data. These data were exported for bioinformatic analyses.

Data Management and Analysis

Data from an OMX imager was received in comma-separated values (.csv) format with plate and well identifiers. These data were annotated with matching chemical and dosage information and compiled in a database. Fold-over-control values for each respective time point were calculated using Eq. (1) for each treatment group:

$$\text{Fold over control} = \mu_{\text{sample}} / \mu_{\text{DMSO}} \quad (1)$$

Curve Fitting Methods

Curve fitting was performed using the well-established “port” algorithm (<http://www.bell-labs.com/project/PORT/doc/port3doc.tar.gz>) for non-linear least squares regression as implemented in the R statistical language (R Development Core Team, 2008). Receptor–ligand interactions were assumed to operate according to Michaelis–Menten kinetics. Therefore, a modified form of the four-parameter logistic model, or Hill slope model [Eq. (2)] (<http://www.ncgc.nih.gov/guidance/section3.html>), was used to determine the Hill slope, E_{MAX} (maximal observed value), E_{MIN} (minimal observed value), and EC_{50} (50% maximal effective concentration). Induction of mRNA occurs downstream of the receptor interaction, and it is likely that other mechanisms have affected the shape of the response for some chemical–gene combinations, suggesting another model may be more appropriate. Using the R^2 values for chemicals that induced genes in this data set, it is clear that this model was appropriate for the vast majority of responses observed.

$$E_{\text{max}} - [(E_{\text{max}} - E_{\text{min}}) / (1 + (x / EC_{50})^{\text{hillslope}})] \quad (2)$$

The R statistical language was chosen for its flexibility of use, ability to handle large amounts of data, and ability to generate large numbers of curves quickly. Only gene–chemical curves that contained a minimum of four

data points were fitted. The lower and upper limits of the search parameters were defined by the minimum and maximum responses observed over the concentration response for an individual chemical. Curves were fitted using five mean data points to obtain the four parameters (E_{max} , E_{min} , EC_{50} , and Hill slope) of the curve. EC_{50} values were bound by the minimum and maximum concentrations.

Reference Chemical (Positive Control) Selection

To determine the appropriate positive control (reference chemical) for each gene target, EC_{50} values, efficacy, and Z-factors were used to determine reference compound candidates for each gene and for both donors at all three time points. The Z-factor is defined in Eq. (3), where σ_s and σ_c represent reference chemical and negative control standard deviations, respectively, and μ_s and μ_c represent reference chemical and negative control maximum fold over control averages, respectively (Zhang et al., 1999).

$$\text{Z-factor} = 1 - [3(\sigma_s + \sigma_c) / |\mu_s - \mu_c|] \quad (3)$$

The reference chemical that consistently produced the largest dynamic range (fold over control response) with a measurable EC_{50} and that had the highest Z-factor was used as the reference compound for a given gene target. Percent efficacy was then determined for all the compounds that generated curves by:

$$\text{Percent efficacy} = [(\text{maximum response}) / (\text{maximum reference response across all time points})] \times 100$$

Response Criteria

Due to the wide and varying nature of the responses observed in these assays (e.g., small vs. large induction/suppression), standard statistical tests were not sufficient to determine the significance of responses. Gene–chemical responses were defined as “hits” according to several data-oriented criteria. The distribution of E_{MAX} values for each gene produced an

exponential growth-like response. This characteristic made the implementation of a global cutoff for all genes unfavorable because this approach assumes linearity. In order to correct for this, the natural log response of the dynamic range ($E_{\max} - E_{\min}$) was calculated for all reference chemicals. This provided a reference range for each gene that all chemicals were then compared to. If a chemical showed greater than a two-fold change from the negative control, and the log-transformed maximum fold change of the test chemical was at least 25% of that observed for the reference compound, then that chemical was defined as a 'hit' for that particular gene and time combination. Genes that did not have a reference chemical with at least a sixfold dynamic range were assessed using an alternative criterion. For these chemical-gene combinations, any response that produced greater than a two-fold dynamic range was defined as a "hit." Using these criteria, concordance data for the blinded internal control compounds were determined using the relationship in Eq. (5):

$$[(\text{Matched})/(\text{matched} + \text{nonmatched})] = (\text{concordance}) \quad (5)$$

***In Vitro–In Vivo* Association Methods**

Comparisons of *in vivo* toxicity data (Martin et al., 2009) with the gene expression data were computed using a relative risk (RR) analysis, specificity, and sensitivity analyses. Both the *in vivo* and *in vitro* numerical values were transformed to a binary format (observed/not observed). The criteria for transforming the *in vivo* data were determined based on whether or not the endpoint was observed for a given chemical. If the endpoint was observed in an *in vivo* toxicity study present in ToxRefDB, then that chemical was considered a positive for that endpoint. The gene expression data were transformed to a binary format according to the 'hit' response criteria defined earlier. The numbers of positives and negatives were tabulated in a 2×2 contingency table.

Permutation Test

In order to identify statistically significant relative risk values, a permutation test was developed. This test randomized which *in vivo* endpoints were associated with each chemical. *In vivo* endpoint data were initially permuted 100 times, in order to generate a distribution of randomly generated relative risk values. If the calculated relative risk value, using real data, fell within the upper 20th percentile, then the permutation test was carried out for an additional 10,000 permutations, to generate a more complete random distribution. If the calculated relative risk fell within the upper 95th percentile of this distribution, then the corresponding assay was selected as being a statistically significant risk factor for the *in vivo* endpoint.

RESULTS

Cultures of primary human hepatocytes from 20,928 wells (96-well plate format) were prepared, cultured, and harvested across 4 time points (0, 6, 24, or 48 h) for a minimum of 5 concentrations of each chemical or positive control (Table 1). Figure 1 contains a summary of the gene targets, receptor pathways, and functional categories assessed by qNPA of the 14 gene targets. The most appropriate reference chemical was determined for each sentinel target gene and associated receptor pathway (Figure 1). Many of the genes were found to be most responsive to PB; however, PB was not always the most efficacious, and rarely the most potent, inducer. For example, the CAR-selective activator CITCO was significantly more potent and less efficacious than PB. The complete data set from this study is available for download from the ToxCast website (<http://www.epa.gov/ncct/toxcast>).

Due to the size of this study (>300,000 data points) and the utility of prototypical receptor activators as efficacious reference chemicals in classifying responses, it was decided to focus our results and discussion primarily on five major pathways shown in Figure 1. Representative concentration-response curves

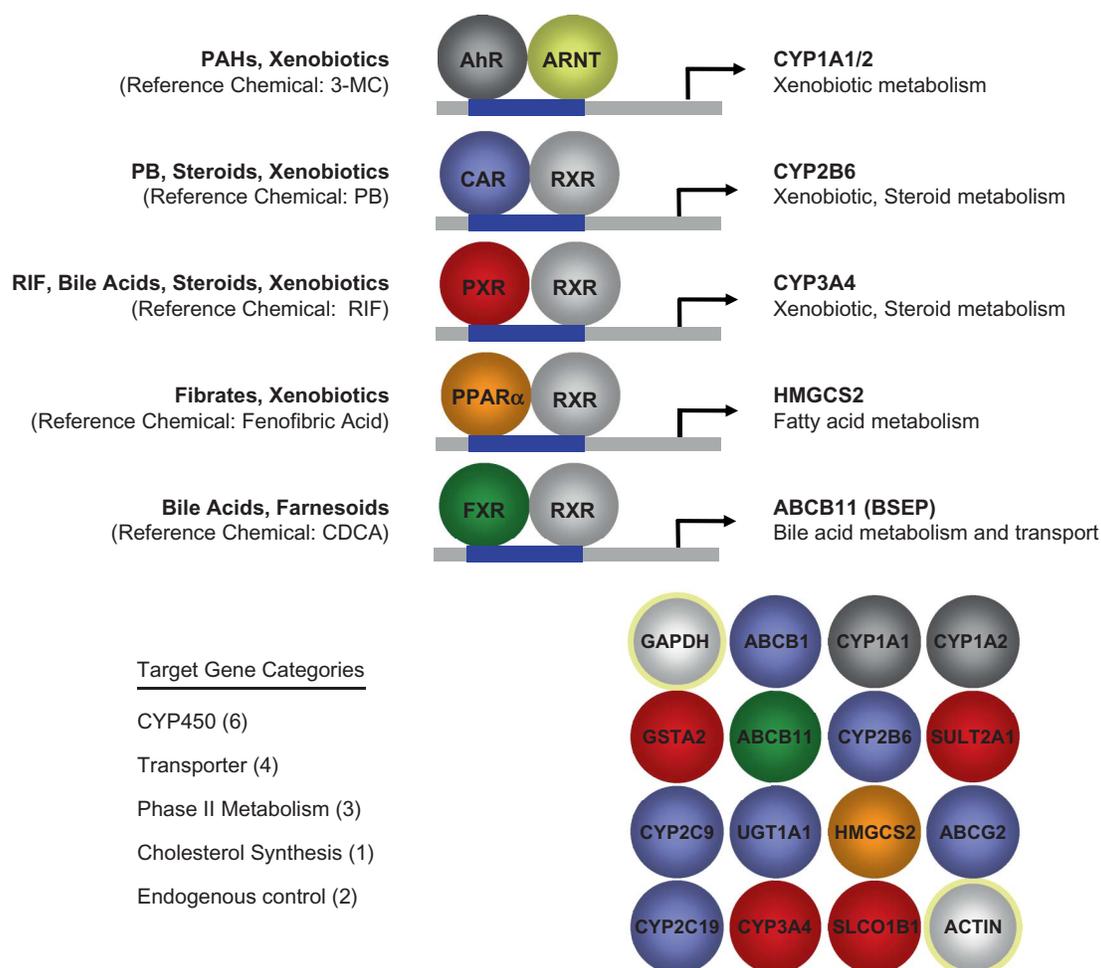


FIGURE 1. (Top) Nuclear receptor pathways summary with corresponding sentinel gene targets and experimentally determined reference chemicals for each pathway. (Bottom) Sixteen-gene array summary depicting the array positions used color coded with the experimentally determined receptor pathway/reference chemical combinations. Endogenous controls GAPDH and β -Actin are shown in white, AhR target genes CYP1A1 and CYP1A2 are shown in gray, CAR target gene CYP2B6 and other PB-responsive genes (e.g., UGT1A1) are shown in blue, PXR target gene CYP3A4 is shown in red along with other gene targets most efficaciously induced by RIF, PPAR α target gene HMGCS2 is shown in orange, and FXR target gene ABCB11 is shown in green.

for each of the gene targets/receptor pathways are shown in Figure 2. The EC_{50} values for each positive control inducer and target gene combination are shown in the figure legends. These data are presented as fold-over-control across a range of concentrations at each time point (6, 24, and 48 h) to portray the temporal relationship of these induction responses. The median Z-factor for 48 h across all of the positive control chemicals and donors was .33, with the individual donors, 776 and 778, producing Z-factors of .17 and .54, respectively. For the AhR pathway (Figure 2A), note that 6 h was sufficient to

observe induction of CYP1A1 and CYP1A2 mRNA expression at approximately 200 fold-over-control, and this response is further induced at the 24-h time point for both genes.

For the CAR pathway (Figure 2, B and C), two positive control inducers (PB and CITCO) were used with CYP2B6 as the sentinel gene. With CITCO, a potent and selective activator of CAR, early induction (6 h) of CYP2B6 that grew to maximal levels by 24 h was noted. An alternative CAR activator, PB (Figure 2C), was observed to be less potent than CITCO but a substantially more efficacious inducer of CYP2B6.

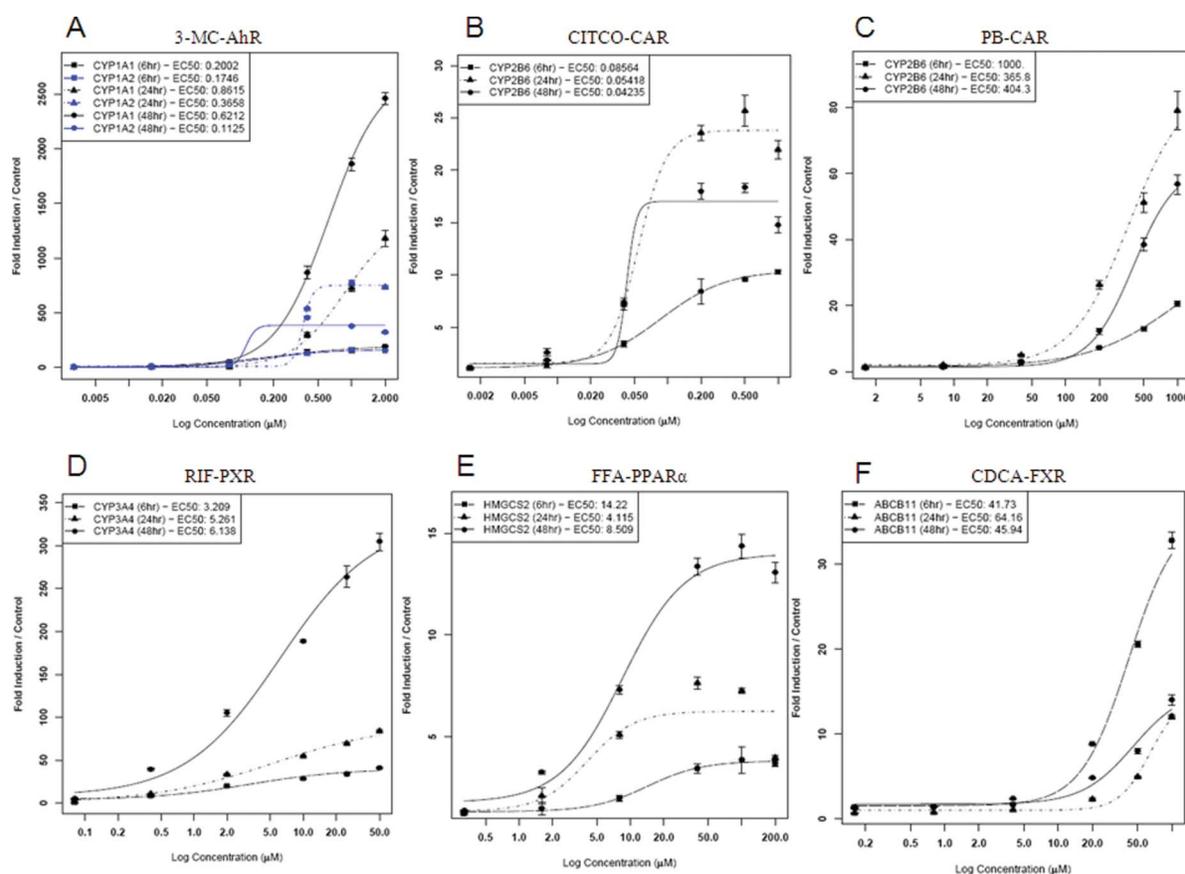


FIGURE 2. Positive control chemical concentration-response curves at each time point (6, 24, and 48 h) for sentinel gene targets in each of the receptor pathways for donor Hu778. (A) CYP1A1 and CYP1A2 curves for AhR agonist 3-MC. (B) CYP2B6 and CYP3A4 curves for CAR selective agonist CITCO. (C) CYP2B6 and CYP3A4 curves with CAR/PXR activator phenobarbital (PB). (D) CYP2B6 and CYP3A4 curves with PXR agonist rifampicin (RIF). (E) HMGCS2 curves with PPAR α agonist fenofibrate (FFA). (F) ABCB11 curves with FXR agonist CDCA. Error bars indicate standard error of the mean for three to four replicates.

The PXR pathway is reflected in the CYP3A4 response to RIF (Figure 2D). Here, concentration- and time-related induction of CYP3A4 was observed with EC₅₀ values in the range of 3–6 μ M. This was somewhat higher than what has been historically reported (approximately 0.2–1 μ M); however, the use of higher RIF concentrations 25 and 50 μ M, where cell health perturbations are often observed, appears to have shifted the concentration-response curve.

Fenofibric acid (FFA) was an effective inducer of the PPAR α gene target HMGCS2 as expected (Figure 2E). FFA also demonstrated CYP3A4 induction at higher concentrations. It is worth noting that there was an unexpected increase in basal expression of HMGCS2 over time in vehicle control cultures, with 10-fold

lower luminescence signal for 0- and 6-h time points relative to the 48-h samples. This was not characteristic of any other gene targets and may be related to an adaptive process within the cultures.

The canalicular bile salt export pump (BSEP or ABCB11) was chosen as the sentinel gene target for the FXR nuclear receptor pathway. Significant induction of ABCB11 was observed with the bile acid and FXR activator CDCA as shown in Figure 2F. This response was markedly more efficacious at the 6-h time point than at the 24- and 48-h time points.

Reproducibility of the Assay Across Chemical Replicates

To assess the reproducibility of the assays, a limited number of identical chemicals,

TABLE 2. Concordance Analysis Performed for the 6-, 24-, and 48-h Time Points Across 14 Gene Targets Assessed by qNPA for The Identical Internal Replicate Chemicals Used in Triplicate

Time	Triplicate compound concordance	Duplicate and triplicate compound concordance	Compound concordance across donor
6 h	36%	34%	64%
24 h	66%	63%	70%
48 h	80%	76%	58%
All time points	65%	60%	63%

Note. Duplicate chemicals used in column 2 were sourced from different vendors. Seven ToxCast chemicals were run across donors and concordance was calculated to determine the feasibility of cross donor comparisons.

assayed in triplicate, were assessed for each gene and time point through concordance analysis which is summarized in Table 2. To avoid inflated concordance values, a stringent approach was adopted in the sense that triplicates were compared pairwise; therefore, if a chemical was a 'hit' in 2/3 triplicates it would receive a concordance value of 33%. This approach was used to develop the most appropriate filtering method to convert concentration-response data to binary yes/no results for each gene/time combination [Eq. (5)]. This analysis demonstrated relatively poor concordance at the 6-h time point when combining all genes (36%) where few responses were observed, while the 24- and 48-h time points had markedly improved reproducibility across the replicate chemicals (66 and 80%, respectively). Unlike the triplicates, the duplicate chemicals were sourced from different vendors and reduced the overall concordance when combined with the triplicate compounds.

Concordance was also assessed on seven chemicals other than positive controls that were run in both donor cell preparations. This analysis provides insight into the variability between the two donors used in this assay. The overall concordance between donors was determined to be 63%, indicating that differences do exist between the 2 donors; however, these differences do not appear drastic enough to prevent adequate comparison of chemicals across donors.

Chemical-Induced Perturbations in Cell Morphology

Cell morphology was monitored as a marker of hepatocyte monolayer integrity. From these observations, both Hu776 and Hu778 hepatocyte preparations produced monolayers of suitable quality to conduct effective studies as supported by the positive control responses with *in vivo*-relevant receptor activators. Following cell morphology across the full concentration profile provided a qualitative platform to evaluate the apparent cytotoxicity of the chemicals at each harvest time point (0, 6, 24, or 48 h). Based on these observations, instances of apparent cytotoxicity were observed for 43 chemicals for at least one concentration/time point. A list of these qualitative assessments is provided in the Supplemental Material (Supplemental File 1). Across the ToxCast chemicals, apparent cytotoxicity was observed with 5 chemicals at or before the shortest time point of 6 h, 18 chemicals produced apparent cytotoxicity by the 24-h time point, and 20 chemicals produced apparent cytotoxicity after 48 h of exposure. The concentrations at which cytotoxicity was initially observed were chemical dependent and spanned the entire concentration range. The median concentration across the data set at which apparent cytotoxicity was first observed is 4 μ M with a mean of 21 μ M. In general, qNPA data generated from time points at or subsequent to observations of apparent cytotoxicity produced no detectable mRNA.

Chemical Efficacy

Figure 3 contains histograms comparing the responses of the ToxCast chemical library to the responses observed by the corresponding reference chemical in regard to efficacy and time in both donors. Histograms, such as 3-MC-induced modulation of CYP1A1 (top left), that were heavily skewed to the left demonstrate the inability of almost any chemical in the ToxCast chemicals to produce a response at or near the efficacy of the reference chemical (3-MC). At 48 h only 6 chemicals produced responses greater than 20% of 3-MC for

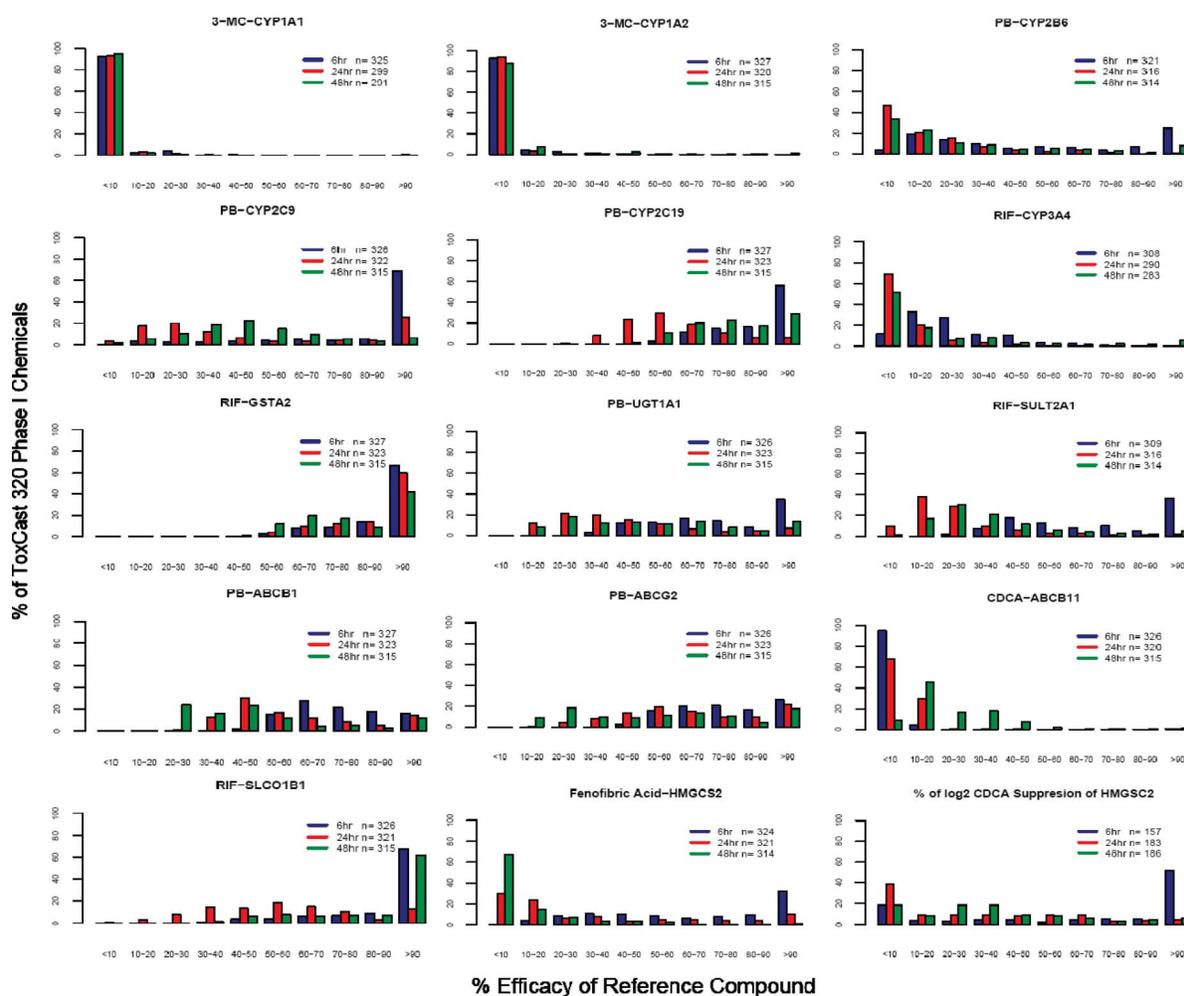


FIGURE 3. Histograms of efficacy for the 320 ToxCast chemicals. The x axis represents the percent efficacy for chemicals relative to positive control chemical response (e.g., a chemical that caused a response >90% of PB for CYP2B6 falls in the far-right column). The y axis represents the percentage of the ToxCast chemicals in the respective efficacy categories.

CYP1A1. Fourteen chemicals at 48 h produced responses greater than 20% of 3-MC for CYP1A2. As expected, due to common regulation by AhR, the distributions for CYP1A1 and CYP1A2 were quite similar in appearance. However, there were some notable differences observed. One difference was a small number of 48-h responses >90% of reference chemical for CYP1A2 and not CYP1A1.

In contrast to the histograms skewed to the left due to large reference chemical responses, other distributions were skewed to the right due to the lack of an efficacious reference chemical (e.g., SLCOB1). GSTA2's response at the 48-h time points produced 113 chemicals with responses >90% of the reference chemical

RIF (1.5- and 2.3-fold). There were 0, 2, and 19 ToxCast chemicals within 90% of CYP2B6's response with treatment of PB at 6, 24, or 48 h, respectively. PB was most efficacious at the 24-h time point, and it appears to require 48 h for many of the ToxCast chemicals to produce a similar response. The two chemicals with responses greater than 90% at 24 h were thiazopyr and dicofol with dynamic ranges of 60.9 (103% of the Hu776 donor response) and 70.6 (91% of the Hu778 donor response) fold-over-control. In addition, some of the ToxCast chemicals produced a greater induction of CYP2B6 than PB. Coumaphos, for example, produced a response of 44-fold-over-control at 24 h, which increased to 121-fold-over-control

(197% of the Hu776 donor response) at 48 h. The phase II conjugating enzyme UGT1A1, thought to be regulated by multiple receptor pathways (e.g., AhR, PXR, and CAR), showed that 112 chemicals had >20% of PB responses, and 6 of these chemicals fell in the >90% category at 6 h. A few chemicals such as propanil and flutidionil produced UGT1A1 induction responses two- to three-fold higher than the PB response (209% and 334% of donor Hu776 response, respectively).

A clear contrast in distributions for the low-efficacy responses (<10% of reference chemical) was observed between the 6- and 24-h distributions compared with that at 48 h. CDCA produced similar ABCB11 responses at 24 and 48 h, with the 6-h responses being approximately twice as efficacious. Therefore, the 6-h response by CDCA was used to determine the percent efficacy of the ToxCast chemicals. After 48 h of treatment with the ToxCast chemicals, the only 2 chemicals that

produced responses >20% of that of CDCA at 6 h were pyraclostrobin and flutolanil. This is consistent with the 6-h data where the reference chemical responses were higher; however, it does not explain the contrast between the 24- and 48-h time points. Data demonstrate that regulation of ABCB11 by the ToxCast chemicals had multifaceted activities with both rapidly and slowly developing phenotypes.

Concentration-Response Curves

Representative concentration-response curves at 48 h for each of the 5 pathways examined in this study were overlaid with the reference chemical curves to demonstrate the relative potency and efficacy of a few of the ToxCast chemicals (Figure 4). CYP1A1 and CYP1A2 induction, reflective of the AhR receptor pathway, are shown following exposure to various ToxCast chemicals and the reference chemical 3-MC. Flumetralin produced the most efficacious CYP1A1 response among the

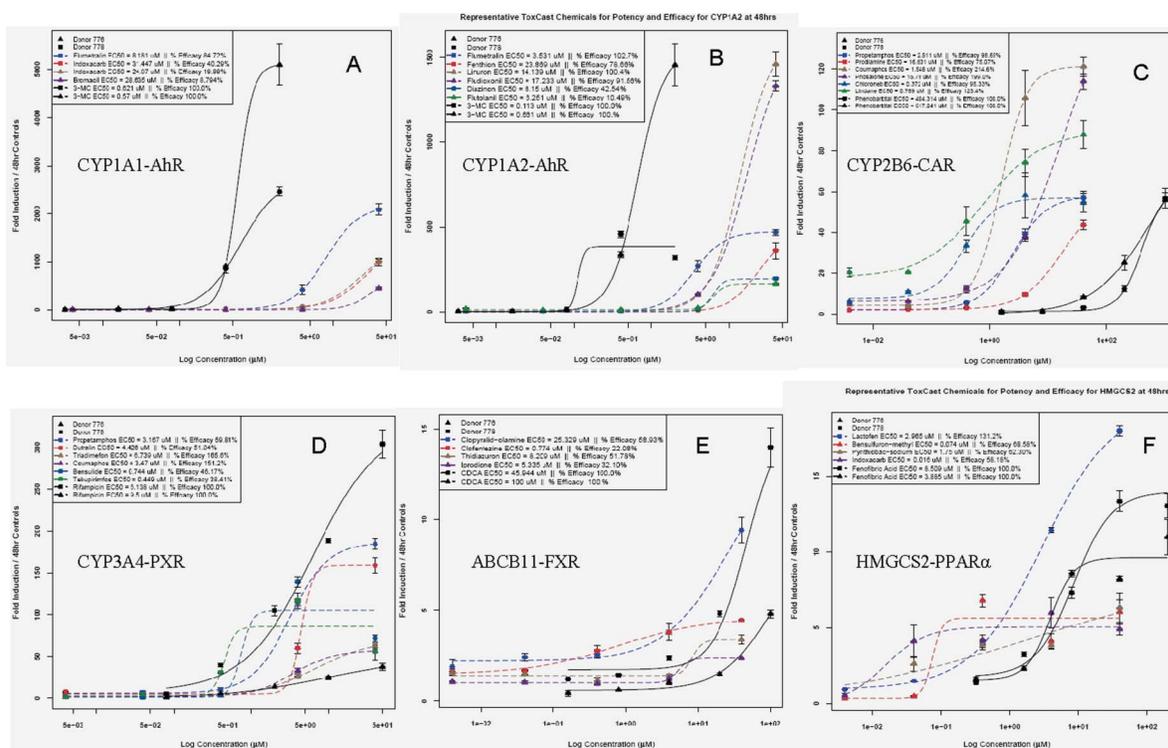


FIGURE 4. Representative concentration response curves demonstrating potency and efficacy of the 320 ToxCast chemicals at 48 h for 5 nuclear receptor pathways. ToxCast chemicals are represented with dashed lines, and reference compounds with solid black lines. Donor 776 data are represented as triangles, and donor 778 as squares. Error bars indicate standard error of the mean for three to four replicates.

ToxCast chemicals with a change of 2084-fold over control. There were significant differences in the response between donors after exposure to 3-MC for both CYP1A1 and CYP1A2. For both genes, donor Hu776 was observed to have a much greater response to 3-MC treatment. Overall these responses at the 48-h time point were markedly less potent than the reference chemical 3-MC responses across the 2 hepatocyte preparations (CYP1A1 EC₅₀ values of 0.581 and 0.663 μ M in hepatocyte preparations Hu778 and Hu776, respectively).

CYP2B6, the sentinel gene for the CAR nuclear receptor pathway, was more potently induced by several ToxCast chemical inducers, with EC₅₀ values ranging from 1.548 to 11.523 μ M relative to the reference chemical PB EC₅₀ value of >200 μ M (Figure 4C). Coumaphos, phosalone, and fludioxinil were assayed in donor Hu776, and were the 3 most efficacious compounds, measuring 197.4, 183.1, and 182.6% of the maximum value reached by PB (24 h). Coumaphos and propetamphos were the most potent of these representative inducers of CYP2B6. Both donors responded similarly to treatment with PB in regard to CYP2B6 induction. CYP3A4 is considered the sentinel gene target for the PXR pathway; however, there is significant cross-talk that takes place among CAR and PXR that can be qualitatively assessed by comparing the relative efficacies of CYP2B6 and CYP3A4 induction (Honkakoski et al., 2003). As a result of this cross-talk, it is not surprising that some of the efficacious inducers of CYP2B6 were also observed to effectively induce CYP3A4 (e.g., propetamphos; Figure 4D). Cases where induction was markedly different (e.g., phosalone with 114-fold induction of CYP2B6 and only 32-fold induction of CYP3A4, and coumaphos [not shown] with 121-fold induction for CYP2B6 and only 56-fold induction for CYP3A4) with respect to efficacy suggest a prominent role for CAR in this response. Compounds such as metolachlor produced a reversed phenotype consistent with a more specific PXR agonist where CYP3A4 induction (81-fold-over-control) was markedly greater than CYP2B6 induction (7.9-fold-over-control) at the 48-h time point.

There was a large disparity between donors after exposure to RIF. CYP3A4 was induced to a maximum of 37-fold in donor Hu776 and 304.5-fold in donor Hu778.

For the FXR pathway, ABCB11 was more responsive, indicated through a larger dynamic range in donor Hu778 in comparison to donor Hu776 (Figure 4E). Clopyralid-olamine was the most efficacious ToxCast chemical inducer of ABCB11 at 48 h, reaching 23.98% of the maximum response observed by the reference chemical, CDCA (6 h). Clopyralid-olamine and CDCA had EC₅₀ values that were not markedly different, indicating that these chemicals may share similar phenotypes with respect to FXR activation.

The fifth nuclear receptor pathway highlighted in Figure 4F is the PPAR α pathway with reference chemical FFA and the target gene HMGCS2. Lactofen was observed to be the most efficacious inducer of HMGCS2, with a 31.2% greater response than FFA and an EC₅₀ of 2.648 μ M. Overall, only 21 of the ToxCast chemicals reached responses greater than 30% of that induced by FFA at 48 h. There were several chemicals that demonstrated an ability to induce HMGCS2 at low concentrations. The most potent HMGCS2 inducer from this group was indoxacarb, with an EC₅₀ of .022 μ M. Indoxacarb and lactofen were also observed to induce multiple gene targets other than HMGCS2, including and CYP1A1 and SULT2A1. It is important to note that 'hit' determination was made respective to donor to correct for variability.

For some gene targets (e.g., SLCO1B1, ABCB1, SULT2A1, UGT1A1), the ToxCast chemicals produced induction two- to three-fold over vehicle controls, while ToxCast chemicals such as mancozeb produced induction responses of five- to six-fold over control for ABCB1 (Supplemental File 1).

Figure 5 demonstrates the number of chemicals meeting the 'hit' criteria by time point. Out of 13,776 possible chemical (excluding positive control compounds), gene, and time assays 2313 met the corresponding 'hit' criteria (16.79%). Out of these 2313 responses, 93% had R^2 values of greater than

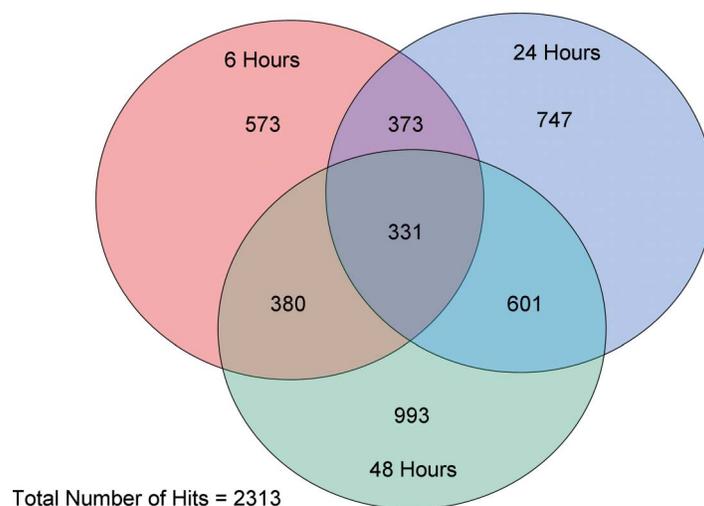


FIGURE 5. Number of ToxCast chemical hits unique or common across time points (6, 24, or 48 h) for expression of 14 genes in human hepatocytes.

TABLE 3. Relative Risk (RR), Sensitivity, and Specificity of Gene Expression After 48-h Exposure for Prediction of the *In Vivo* Chronic Toxicity of ToxCast Chemicals

Gene	Endpoint	True positive count	False positive count	True negative count	False negative count	RR	Sensitivity	Specificity	Permuted <i>p</i> value
CYP3A4	Rat thyroid tumors	22	87	74	7	2.34	0.76	0.46	0.02
CYP2B6	Rat thyroid tumors	23	100	61	6	2.09	0.79	0.38	0.04
SULT2A1	Rat thyroid hyperplasia	10	42	125	13	2.04	0.43	0.75	0.04
HMGCS2	Rat proliferative liver lesions	7	5	126	52	2.00	0.12	0.96	0.04
SLCO1B1	Rat liver tumors	4	4	165	17	5.35	0.19	0.98	0.01
HMGCS2	Rat liver tumors	4	8	161	17	3.49	0.19	0.95	0.03
CYP2B6	Rat liver hypertrophy	49	74	54	13	2.05	0.79	0.42	0.00
CYP2B6	Rat liver apoptosis necrosis	17	106	63	4	2.32	0.81	0.37	0.03

Note. Three of the genes represent chemical effects on CAR (CYP2B6), PPAR α (HMGCS2), and PXR (CYP3A4) receptor pathways. SULT2A1 is a phase II enzyme and SLCO1B1 is a transport protein. The six toxicity endpoints displayed are a selected few with statistically significant relative risk responses greater than or equal to 2 for at least one gene/pathway.

or equal to .7. As expected, the number of “hits” increased as time passed, resulting in 573 “hits” at 6 h, 747 “hits” at 24 h, and 993 “hits” at 48 h. There were 331 chemical–gene combinations that ‘hit’ all three time points.

Association of *In Vitro* (Human) Gene Expression to *In Vivo* (Rodent) Toxicity

The qNPA results were compared to data in ToxRefDB from *in vivo* chronic rodent studies

using relative risk (RR), sensitivity and specificity analyses. The eight *in vivo* categorized endpoints that produced statistically significant associations with the gene induction data were rat liver tumors, rat liver apoptosis/necrosis, rat liver hypertrophy, rat proliferative liver lesions, rat thyroid hyperplasia, rat thyroid tumors, rat tumorigen, and rat multigender tumorigen. Table 3 provides the data from the relative risk, sensitivity, and specificity analyses for 7 genes

regulated by the AhR, CAR, PXR, and PPAR α receptors at 48 h. A relative risk (RR) of 3.49 was calculated for the formation of rat liver tumors by ToxCast chemicals causing induction of the PPAR α sentinel gene HMGCS2. Permutation analysis demonstrated the statistical significance of this result at the $p < .05$ level. For the 21 current ToxCast chemicals, with evidence of rat liver tumors captured in the ToxRefDB, 4 were identified as "hits" (true positives) for HMGCS2, while 17 did not meet the 'hit' criteria (false negatives). 165 of the 309 ToxCast chemicals were identified as true negatives.

CYP2B6 induction, was associated (RR > 2) with rat liver hypertrophy, rat thyroid tumors, and rat liver apoptosis/necrosis with RR values of 2.05, 2.09, and 2.32, respectively. Permutation analysis demonstrated the statistical significance of these results at the $p < .05$ level. When examining the 29 chemicals in ToxRefDB that produced thyroid tumors in rats, 23 were considered to be CYP2B6 "hits" in this assay (true positives) and 6 did not meet the set criteria (false negatives) (Supplemental File 2). Sixty-three (63) chemicals were observed to produce rat liver hypertrophy. Of those 63, 49 were found to associate with "hits" for CYP2B6 at the 48-h time point, and 13 were found to be false negatives. In addition, of the 13 false negatives, 2 of the chemicals produced "hits" at the 24- and 6-h time points, suggestive of possible time-dependent processes for these chemicals (e.g., autoinduction) that may lead to tolerance observations with longer time points. Of the 21 chemicals that produced rat liver apoptosis/necrosis, 17 were regarded as CYP2B6 "hits" with 4 false negatives.

Another notable RR observation (RR = 2.35) from Table 3 was that of rat thyroid tumors associated with chemicals found to induce CYP3A4 expression, reflective of PXR pathway activation. Permutation analysis demonstrated the statistical significance of this result at the $p < .05$ level. Here 22 out of the 29 chemicals found to produce rat thyroid tumors induced CYP3A4, while 7 chemicals did not meet the 'hit' criteria (false negatives).

Induction of another PXR regulated gene, SULT2A1, was associated with rat thyroid hyperplasia with an RR value of 2.04. Ten out of 167 CYP3A4 inducible genes as true negatives. Permutation analysis demonstrated the statistical significance of this SULT2A1 result at the $p < .05$ level.

The transporter, SLCO1B1, was statistically significant in RR analysis with rat liver tumors, with an RR of 5.35 at the 48-h time point. SLCO1B1 also had a RR of 3.12 at 24 h; however, this result was not statistically significant based on the low number of true positives, producing an increased likelihood in a high RR result.

DISCUSSION

In the present study, the ToxCast chemicals were characterized in cultures of primary human hepatocytes using qNPA and cell morphology assessments. Concentration-response curves for each of the ToxCast chemicals were generated to derive relative potency and efficacy data. Associations were observed between the five major receptor pathway responses and a limited number of *in vivo* rodent endpoints and demonstrated the utility of these assays that are being incorporated into the larger ToxCast predictive modeling effort (<http://www.epa.gov/ncct/toxcast>).

Concordance analysis for each time point on the replicate chemicals to assess the reproducibility of the assays demonstrated that the 6-h data were less concordant than the 24- and 48-h time points. These results are consistent with the fact that induction of gene expression is a time-dependent process, and sufficient time is required to allow treatment-related effects to gain momentum and overcome constitutive expression or feedback mechanisms. The triplicate chemicals were mostly inactive across many of the genes, making a comprehensive concordance analysis difficult. It is clear that many chemicals, including the replicates, only showed induction near the high end of the concentration-response range tested, often limiting the ability to confidently assign a potency value. If we had tested to a

higher concentration ($>40 \mu\text{M}$), had additional donors and concentrations, or had selected replicate chemicals that were more potent and efficacious, it might have been possible to generate more robust concentration response data and improve the outcome of the concordance analysis. Nonetheless, overall these data demonstrated reliable reproducibility of the assay, especially at the 48-h time point.

For the CAR pathway, PB and CITCO induced CYP2B6 and CYP3A4 as expected, with efficacies for CYP2B6 larger than CYP3A4. These data were consistent with CITCO's relative affinity/efficacy to activate CAR with higher affinity than PXR. Interestingly, the temporal profiles with the selective CAR activator CITCO show the induction of CYP2B6 mRNA was quite rapid when compared with the selective PXR agonist RIF. For RIF, both CYP2B6 and CYP3A4 were induced, and the induction through time increased up to the 48-h time point. FFA induction of CYP3A4 was consistent with literature reports indicating that higher concentrations of FFA can activate PXR (Prueksaritanont et al., 2005). Reference chemical responses demonstrated the fidelity of the human hepatocyte cultures as *in vitro* models for human liver.

From the chemical efficacy distributions in Figure 3, only a handful of ToxCast chemicals showed CYP1A induction comparable to the reference chemical 3-MC. In contrast, the distribution observed with CYP2B6, the most frequently induced gene of the 14 target genes assessed, as well as UGT1A1 indicates a broad range of efficacies across the ToxCast chemicals. The promiscuous and efficacious inducibility of UGT1A1 observed is consistent with the literature demonstrating multiple receptor pathways such as AhR or CAR thought to regulate its expression. A notable observation that ABCB1 was most efficaciously induced by PB rather than RIF, along with the observation that multiple ToxCast chemicals caused responses $>100\%$ of the PB-mediated induction of ABCB1, suggests that there is more to learn about the transcriptional regulation of this important efflux transporter.

As proof of concept for the predictive value of these *in vitro* assays, associations of mRNA data were examined with chronic *in vivo* rodent studies using RR analyses. This cross-species comparison was driven by the fact that the *in vivo* toxicity studies for all of these chemicals were conducted in rodents and other animal species. However, these same animal study results are extrapolated for human health risk assessments for these same chemicals, so associations across species would be expected if this human *in vitro* assay was also predictive of human toxicity. For some of the chemicals, effects on ABCB1, CYP1A1, CYP2B6, CYP3A4, HMGCS2, SULT2A1, and SLCO1B1 expression produced RR factors greater than 2 at either the 24-h or 48-h time points, indicating predictive power. The association between CYP2B6 and rat thyroid tumors was interesting when accounting for the recent advances in the understanding of CAR and its role in thyroid hormone regulation (Konno et al., 2008). While it is not clear what role CYP2B6 may play in the regulation of thyroid homeostasis, CAR also regulates UGT1A1 and SULT2A1, both of which are involved in the glucuronidation and sulfation of thyroxine (T_4) (Maglich et al., 2004; Ebmeier & Anderson, 2004). Due to the multiple pathways by which UGT1A1 may be regulated in addition to CAR, including AhR or PXR, it is understandable that a direct association was not observed between UGT1A1 and thyroid tumors. The association of HMGCS2 with rat liver tumor formation was consistent with literature reports linking PPAR α activators and tumor formation in rat liver (James et al., 1998). Humanized PPAR α mice were shown to lack the tumor phenotype produced in wildtype mice, therefore it is not clear whether the prediction of rat liver tumors is a risk factor for humans (Gonzalez & Shah, 2008). In the case of CAR and PPAR α activators, as with all interspecies comparisons, it is important to note that these species differences occur at the molecular levels and lead to profound differences in metabolism and molecular signaling pathways, which may limit the utility of these interspecies correlation approaches. Overall, the RR assessments

revealed statistically significant relationships between a limited number of *in vivo* endpoints and several genes significant to liver function and representative of five major receptor pathways. Further studies relating these data to other ToxCast assays measuring binding, agonism, and antagonism in these same receptor pathways will likely improve the ability to predict *in vivo* toxicity.

The current data set demonstrates the ability of some chemicals to alter gene expression, and by inference biological pathways, at relatively low concentrations in human hepatocytes. For example, the ametryn induction of HMGCS2 with an EC_{50} of 0.060 μM and E_{max} of 5.1-fold over control suggests activation of the PPAR α signaling pathway. Chloroneb induction of CYP2B6 with an EC_{50} of 0.36 μM and E_{max} of 58-fold over control where minimal CYP3A4 induction was observed suggests activation of CAR. The biological, let alone the toxicological, significance of these *in vitro* effects on gene expression in human hepatocytes remains to be determined.

While it is possible that changes in gene expression and activation of nuclear receptor signaling pathways represent key events in a toxicological mode of action, whether that is the case for any of the genes, receptors, and chemicals in the present study has not been confirmed. One approach to evaluate the relevance of these *in vitro* data is "reverse" dosimetry. By measuring intrinsic clearance in human hepatocytes and human plasma protein binding, pharmacokinetic models can be calibrated in order to predict equivalent oral doses for humans that might result in steady-state tissue concentrations comparable to the EC_{50} values reported in the present study. This intrinsic clearance data, if related to gene expression, may provide valuable insight into the role of metabolic activation/deactivation with corresponding toxicity phenotypes. This approach would be an initial step toward defining the *in vivo* exposure-dose-toxicity relationships for ToxCast chemicals relative to *in vitro* bioactivity, and will aid in interpretation of high-throughput

screening results. Interpretation of these results in the context of human exposure will provide guidance on using *in vitro* data for predictions of *in vivo* toxicity and chemical prioritization.

In conclusion, the bioactivity of the 309 unique chemicals currently in the ToxCast library was characterized in cultures of primary human hepatocytes over concentration and time-course measurements. Many of these chemicals induced the expression of these human genes, providing distinct bioactivity profiles that may be useful in classifying and ranking these chemicals based on their potential to impact xenobiotic metabolism pathways regulated by nuclear receptors and often associated with toxicity. In addition, direct associations were observed between the activation of key human receptor pathways and specific and relevant rodent *in vivo* toxicity endpoints. These findings indicate the potential of metabolically competent, *in vitro* hepatocyte culture systems for generating data useful in predictive toxicity modeling, and identify putative human toxicity pathways for specific disease endpoints.

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