Impact of Environmental Chemicals on Key Transcription Regulators and Correlation to Toxicity End Points within EPA's ToxCast Program

Matthew T. Martin,*,† David J. Dix,† Richard S. Judson,† Robert J. Kavlock,† David M. Reif,† Ann M. Richard,† Daniel M. Rotroff,‡ Sergei Romanov,|| Alexander Medvedev,§ Natalia Poltoratskaya,§ Maria Gambarian,§ Matt Moeser,§ Sergei S. Makarov,§ and Keith A. Houck†

National Center for Computational Toxicology, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina, Department of Environmental Sciences and Engineering, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, and Attagene Inc., P.O. Box 12054, Research Triangle Park, North Carolina

Received September 9, 2009

Exposure to environmental chemicals adds to the burden of disease in humans and wildlife to a degree that is difficult to estimate and, thus, mitigate. The ability to assess the impact of existing chemicals for which little to no toxicity data are available or to foresee such effects during early stages of chemical development and use, and before potential exposure occurs, is a pressing need. However, the capacity of the current toxicity evaluation approaches to meet this demand is limited by low throughput and high costs. In the context of EPA's ToxCast project, we have evaluated a novel cellular biosensor system (Factorial[1]) that enables rapid, high-content assessment of a compound's impact on gene regulatory networks. The Factorial biosensors combined libraries of *cis*- and *trans*-regulated transcription factor reporter constructs with a highly homogeneous method of detection enabling simultaneous evaluation of multiplexed transcription factor activities. Here, we demonstrate the application of the technology toward determining bioactivity profiles by quantitatively evaluating the effects of 309 environmental chemicals on 25 nuclear receptors and 48 transcription factor response elements. We demonstrate coherent transcription factor activity across nuclear receptors and their response elements and that Nrf2 activity, a marker of oxidative stress, is highly correlated to the overall promiscuity of a chemical. Additionally, as part of the ToxCast program, we identify molecular targets that associate with *in vivo* end points and represent modes of action that can serve as potential toxicity pathway biomarkers and inputs for predictive modeling of *in vivo* toxicity.

Introduction

Estimating the toxicity of environmental chemicals is impeded by the sheer number of potential contaminants, the high costs of animal testing, and the poor prognostic power of traditional toxicity testing for assessing risks to humans. Relatively few predictive *in vitro* assays have been routinely used for screening, outside of those for genetic toxicology or specific molecular targets such as the steroid hormone receptors and the sodium channel hERG (*1, 2*). The paradigm of traditional toxicology has been challenged to shift toward using predictive or pathway-based toxicology approaches to more efficiently and systematically evaluate large numbers of chemicals for a diversity of toxicity end points (*3*). The U.S. Environmental Protection Agency (EPA) and National Institutes of Health (NIH) have responded to this challenge with major new computational toxicology testing and research initiatives. EPA's ToxCast program (*4*), and the affiliated Tox21 program (*5*), the latter a collaboration between three U.S. government entities, EPA, NIH's National Toxicology Program, and the NIH Chemical Genomics Center (NCGC), are generating broad spectra of high-throughput/high-content biochemical and cell-based *in vitro* assays for a relatively large number of environmental chemicals (*5, 6*). These data are being used to derive biological and chemical profiles predictive of *in vivo* biological end points and to serve as the basis for new toxicity screening and prioritization approaches.

One source of high-content, cellular data was obtained using a recently developed biological profiling technology, Factorial (*7*), that enabled high-content, functional assessment of core components of cellular gene regulatory networks. Assessment was accomplished by measuring the activity of transcription factors (TFs), i.e., the specialized classes of DNA-binding proteins that recognize regulatory elements in gene promoters and control transcription. Originally designed for multiplexed detection of specific cis-regulatory response element constructs (CIS), the technology has been further developed to provide assessment of the trans-activating potential of multiple nuclear hormone receptors, a superfamily of ligand-activated TFs (TRANS). The current technology was utilized in phase I of EPA's ToxCast program to screen 320 environmental substances, the large majority of which are pesticide actives. We report here the results of that screening using 48 CIS and 25

* To whom correspondence should be addressed. Phone: 919-541-4104. E-mail: martin.matt@epa.gov.
† U.S. Environmental Protection Agency.
‡ University of North Carolina at Chapel Hill.
§ Attagene Inc.
|| Present address: Nanosyn Biology, Capitola Dr. 800, Durham NC 27713.
[1] Factorial is a trademark of Attagene Inc.

10.1021/tx900325g
Published on Web 02/10/2010

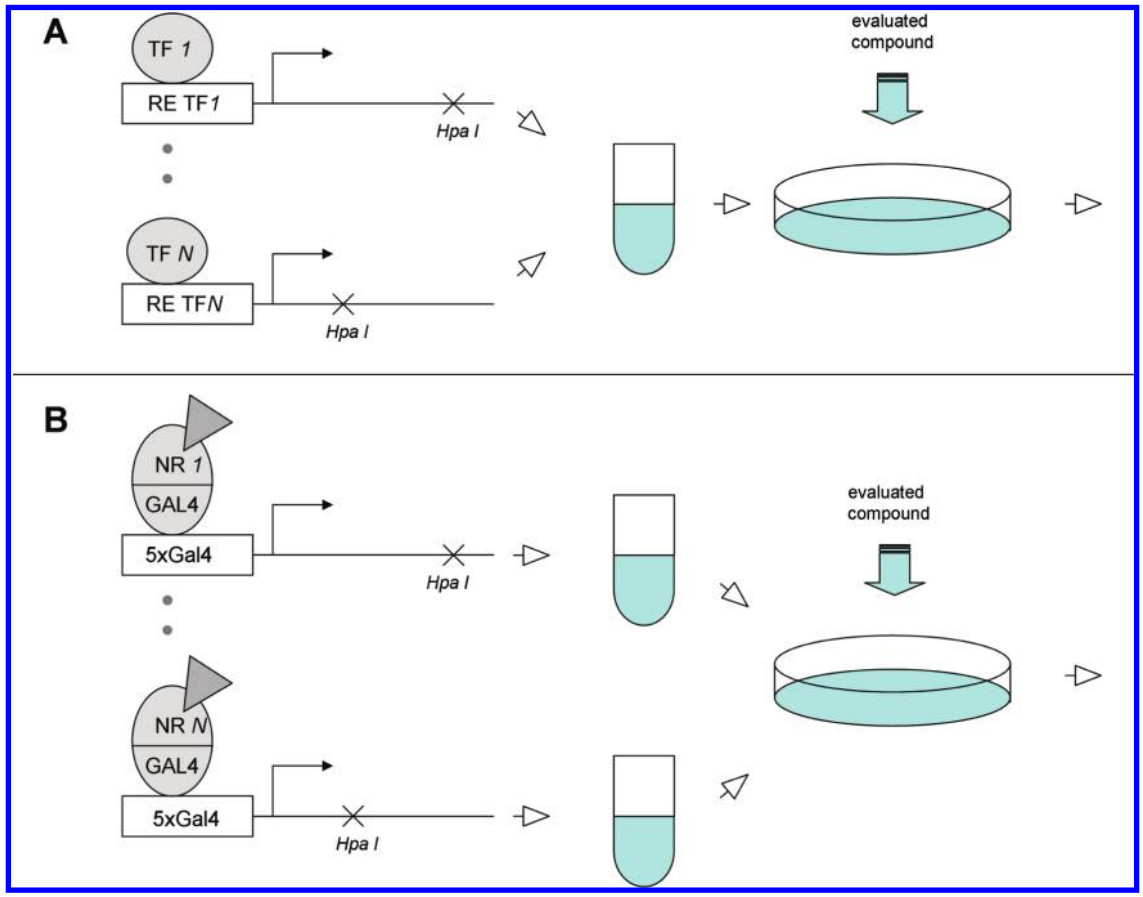

Figure 1. Evaluation of ToxCast compounds by using libraries of cis- and trans-reporter transcription units. Schematic representation. (A) Detection of cis-RTUs. A mix of 51 individual plasmids encoding cis-RTUs was cotransfected into a suspension of HepG2 cells, cells were plated, and stimulated with evaluated compound. At the end of the incubation period, total RNA was isolated and detected as described previously (*8*). (B) Detection of trans-RTUs. Twenty-four trans-RTU plasmids were separately transfected into HepG2 cells and the transfected cells pooled, plated, and stimulated with evaluated compound. At the end of incubation, total RNA was isolated and detected as described previously (*8*).

TRANS assays chosen to place particular emphasis on factors and receptors that control toxicologically relevant cellular responses to xenobiotics, genotoxic stress, hypoxia, oxidative damage, immuno-modulation, and endocrine disruption. Furthermore, we use these biological profiling results to identify perturbed gene regulatory networks and possible modes of action for the ToxCast 320 environmental substances. By introducing the plurality of CIS and TRANS assays into a human liver cell line, the biosensors enabled the characterization of chemicals in the context of the cell's gene regulatory networks. We show that HepG2 cells responded to many chemical compounds through changes at the level of transcription factor and nuclear receptor activities. The changes elicited by the chemicals reflect well-understood interactions in some instances, whereas in others, they provide new insights into possible modes of action for chemical specific toxicities.

Materials and Methods

Chemical Library. The ToxCast 320 chemical library consists of 309 unique chemical structures meeting physicochemical property requirements for high-throughput screening (*8, 9*). 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 EPA. These data were extracted from documents, standardized, and compiled in the EPA ToxRefDB relational database (*10*−*13*). The derivation of the mammalian *in vivo* toxicity end points was focused on chronic/cancer, multigenerational reproductive, and prenatal developmental toxicity studies. For any given study type, roughly 250 of the 309 unique chemicals tested in this study have toxicity end point data in ToxRefDB. The full list of chemicals is available with quality reviewed structure annotation from the EPA DSSTox Web site (*8*). Chemicals samples were procured and plated by BioFocus DPI (San Diego, CA). Supplier-provided certificates of analysis indicated purity of >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 LC and GC/MS (liquid and gas chromatography−mass spectrometry), subsequent to assay screening, has 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 QC information mapped to chemical sample and solution IDs will be provided on the ToxCast Web site as an auxiliary chemical file (*4*). Compounds were dissolved in dimethyl sulfoxide (DMSO) to a final target concentration of 20 mM, in almost all cases. For testing in concentration−response format, serial dilutions were performed in DMSO followed by aqueous dilution in cell culture medium.

Assay Design and Implementation. Attagene Inc. (RTP, NC), under contract to the U.S. EPA (Contract Number EP-W-07-049), provided multiplexed reporter transcription unit (RTU) assays (Factorial, patents pending) consisting of 48 human transcription factor DNA binding sites transfected into the HepG2 human liver hepatoma cell line as previously described (*7*). In addition to the cis-acting reporter genes (CIS), a modification of the approach was used to generate a trans-system (TRANS) with a mammalian one-hybrid assay consisting of an additional 25 RTU library reporting the activity of nuclear receptor (NR) superfamily members (Table S-1, Supporting Information) (*14*). A schematic representation of

both systems is illustrated in Figure 1. The human ligand-binding domain of each nuclear receptor was expressed as a chimera with the yeast GAL4 DNA-binding domain that activated in trans a 5X-UAS-TATA promoter, which regulated the transcription of a reporter sequence unique to each NR RTU. To ensure the specificity of detection, each individual trans-RTU system including both receptor and reporter gene was separately transfected into suspended cells followed by pooling and plating of the transfected cells prior to screening. A major difference between the CIS and TRANS system is that in CIS activities of endogenous TFs are measured, whereas the TRANS assay evaluates changes in activities of exogenous, chimeric NR-Gal4 proteins. Since the HepG2 cell line does not express some nuclear receptors, the CIS assay cannot be used to evaluate these targets.

Concentration Selection. An initial cytotoxicity screen was performed to establish the maximum tolerated concentration (MTC) of the chemical library. The chemicals were tested for cytotoxicity against HepG2 cells in the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay (*15*) following 24 h chemical exposure to five concentrations with an upper concentration of 50 μM and 10-fold dilutions. All concentrations were run in triplicate. IC_{50} values were determined on the basis of 50% cell death, as measured by decreasing MTT conversion to formazan. The MTC was derived on the basis of one-third the calculated IC_{50}, or if no IC_{50} was determined, the MTC was set to 100 μM. Results of cytotoxicity assessment are shown below.

Chemicals were then tested in the CIS and TRANS assays at seven concentrations starting at the MTC and followed by 3-fold serial dilutions. Following exposure to chemical for a 24 h period, cells were harvested, and RNA was isolated and reverse transcribed into cDNA using fluorescent-tagged primers. Digestion with *Hpa*I resulted in unique reporter gene fragments that were resolved and quantitated by capillary electrophoresis as previously described (*7*).

Statistical Analysis. Changes in transcription factor activity were expressed as fold-change over the DMSO control by dividing expression levels of treated by the average of control (DMSO) treatments from the same assay plate. Both induction and suppression can occur. However, only 16 out of >20,000 possible chemical-assay combinations had marked suppression and were observed at no more than two chemicals per assay. Therefore, suppression values were filtered out and only induction levels were considered in subsequent analyses. An initial maximum fold-change value (*Emax*) was used to compare across assays. Hierarchal clustering was performed using log 2-transformed *Emax* and subjected to clustering by Pearson's Dissimilarity using Ward's Method. Analyses were carried out using R version 2.8.1 (*16*). *Emax* values were also used to determine an optimal global cutoff as one criterion in establishing a hit, on the basis of the agreement between replicates and stabilization of the overall hit rate as the *Emax* filter increases (Figure S-1, Supporting Information). Specifically, overall and hit concordances were calculated as is done in the final replicate analysis shown below, except solely using an *Emax* filter to establish hit calls. For each pair in the triplicate sets (comparing A with B, B with C, and A with C), we asked if the chemicals were both hits, both nonhits or if they disagreed. Total concordance was defined as all 3 were hits or all 3 were nonhits and nonconcordance as either 1 or 2 were hits and the remainder were nonhits. The total overall concordance is the number of comparisons where the pair was either hits or both nonhits, divided by the total number of comparisons. The hit concordance is the number of cases where each of the pair was a hit divided by all cases where one or each of the pair was a hit.

The pairwise correlation of *Emax* values between any two assays was represented by R^2. The distribution of correlations across all pairwise combinations of assays, a total of 2628, determined the ninety-fifth and ninety-ninth percentile, R^2 of 0.27 and 0.51, respectively. These values help determine the relative significance of the correlation between any two assays in this data set.

A Hill function was fit to all fold-change data and an AC50 was derived, the concentration in which 50% of the maximal response, based on the fitted curve, is achieved. All assay−chemical combinations that did not achieve a fit with an R^2 >0.5 and an *Emax* greater than two were considered negative. A hit is considered anything that achieved an AC50 and met the criteria described above. A univariate analysis was conducted in order to test for associations, by way of relative risk (RR) values, between *in vitro* assays and mammalian *in vivo* toxicity end points. The mammalian *in vivo* toxicity end points, from chronic/cancer rat and mouse, multigeneration rat, and prenatal developmental rat and rabbit toxicity studies, were previously compiled and aggregated for use in predictive toxicology (*10*−*12*). Each chemical−end point combination assessed in a toxicity study and captured in ToxRefDB were represented as the lowest effect level (LEL: mg/kg/day) if the end point was observed, thus defining a positive for an *in vivo* end point. RR values were calculated as follows:

$$RR = [TP/(TP + FP)]/[FN/(TN + FN)]$$

where TP and FP represent the numbers of true and false positive chemicals, respectively, and TN and FN represent the numbers of true and false negative chemicals, respectively. A permutation test was developed in order to identify significant RR values and was carried out for 10,000 permutations. If the RR fell within the upper 95th percentile ($p < 0.05$) of the permuted data, then the corresponding association was regarded as statistically significant. The permuted RR percentile values for all *in vitro* assays by *in vivo* end points were subjected to clustering by Pearson's Dissimilarity using Ward's Method.

Results

Cytotoxicity Assessment. Prior to running the CIS and TRANS assays, cytotoxicity was assessed in an attempt to establish a MTC for each chemical for purposes of concentration selection. Of the 320 total tested chemicals, 246 (77%) were not frankly cytotoxic under conditions of the assay, and therefore, a MTC of 100 μM was used for subsequent testing. Sixty-four chemicals (20%) showed cytotoxicity at micromolar concentrations, whereas another 10 chemicals (3%) displayed cytotoxicity at submicromolar concentrations.

Summary Statistics. In total, there were 23,360 chemical−assay observations with 1,923 (8.2%) showing significant responses. Of these hits, 78% of the AC50 were greater than 10 μM with 4% being under 1 μM. The median number of chemical hits across all assays was six, with a comparable hit rate across both CIS and TRANS formats. The pregnane X receptor response element (PXRE) was the most active of the assays with 234 significant chemical interactions (225 unique chemicals), consistent with the promiscuous nature of the receptor, which is known to be activated by a wide range of pharmaceuticals, steroids, xenobiotics, and natural products (*17*). A complete listing of the assays with the number of hits per assay along with the maximal responder across the ToxCast 320 chemical library, and the *Emax* of the positive control, where available, is presented in Table 1. In the TRANS system, individual members of the TF family can be distinguished, for example, PPARα, PPARγ, and PPARδ, whereas the CIS system evaluates the integral activity of the entire PPAR family through use of the direct repeat (DR)-1 PPAR response element (PPRE) which does not show receptor isotype specificity (*18*). In total, 10 NR families across 16 TRANS assays were represented with their corresponding response elements.

Replicate Analysis. Of the 320 chemicals tested for transcription factor modulation, there are three triplicates and five duplicates imbedded into the blinded chemical set. The triplicates were from the same production lot and vendor, whereas the duplicates were sourced independently and therefore excluded from the replicate analysis. Pairwise assessments of the

Table 1. 73 CIS and TRANS Assays Listed with the Number of Hits Per Assay, the Maximal Responder out of the ToxCast_320 Chemical Library, and Positive Control (Where Available)[a]

CIS/TRANS related assays				remaining CIS assays				remaining TRANS assays			
assay Name	no hits (n = 320)	ToxCast_320 Max responder (*Emax*)	positive control (*Emax*)	assay name	no hits (n = 320)	ToxCast_320 Max responder (*Emax*)	positive control (*Emax*)	assay Name	no hits (n = 320)	ToxCast_320 Max responder (*Emax*)	positive control (*Emax*)
ERE_CIS	39	Pyridaben (8.7)	Estradiol (9.3)	Ahr_CIS	54	Tetraconazole (72)	FICZ (100)	ERRa_TRANS	3	Cyazofamid (2.1)	
ERa_TRANS	90	Pendimethalin (27)	Estradiol (23) 6-Fluor-Test (23)	AP_1_CIS	52	Prochloraz (4.6)		ERRg_TRANS	13	Fenthion (2.5)	
IR1_CIS	3	Tebupirimfos (3.5)		AP_2_CIS	0	Hexaconazole (1.7)		RXRa_TRANS	0	Esfenvalerate (2.3)	
FXR_TRANS	1	Butachlor (2.3)	CDCA (6.5)	NRF2_ARE_CIS	165	Dichlorvos (16)		RXRb_TRANS	8	Fludioxonil (17)	
GRE_CIS	0	Spiroxamine (1.6)	Dexamethazone (5.0)	BRE_CIS	93	Fipronil (12)		AR_TRANS	0	Butachlor (2.1)	6-Fluor-Test (6.3)
GR_TRANS	0	Butachlor (1.9)		C_EBP_CIS	5	Cyazofamid (2.4)		Hpa5_TRANS	0	Rotenone (1.9)	
DR4_LXR_CIS	7	Tebupirimfos (3.8)		CMV_CIS	37	Tebupirimfos (3.4)		HNF4a_TRANS	16	Trichlorfon (3)	
LXRa_TRANS	23	Tebupirimfos (23)	T9 (55)	CRE_CIS	52	Prallethrin (4.4)	Forskolin (2.5)	NURR1_TRANS	9	Pirimiphos-methyl (5.6)	
LXRb_TRANS	21	Tebupirimfos (13)	T9 (81)	E2F_CIS	0	Triclosan (1.5)		THRa1_TRANS	18	(Z,E)-Fenpyroximate (3.2)	T3 (11)
PPRE_CIS	125	Resmethrin (5.7)	Rosiglitazone (6.7)	E_Box_CIS	1	Prallethrin (2.5)					
PPARa_TRANS	9	Lactofen (11)	GW7647 (11)	EGR_CIS	40	Prallethrin (8.1)					
PPARd_TRANS	1	Flusilazole (2)	GW7647 (13)	Ets_CIS	0	Fluazinam (1.3)					
PPARg_TRANS	146	Resmethrin (22)	GW7647 (39) Rosiglitazone (31)	FoxA2_CIS	0	d-cis,trans-Allethrin (1.8)					
PXRE_CIS	234	Flufenacet (31)	Rifampicin (29)	FoxO_CIS	0	Simazine (1.5)					
PXR_TRANS	102	Fipronil (7.9)		GATA_CIS	0	Rotenone (1.4)					
DR5_CIS	27	Imazalil (3.2)	9-cis-Retinoic Acid (7.5)	GLI_CIS	2	d-cis,trans-Allethrin (3)					
RARa_TRANS	49	Lindane (5.9)		HIF1a_CIS	25	Tetraconazole (3.7)					
RARb_TRANS	5	Oxadiazon (2.6)		HNF6_CIS	0	Spiroxamine (1.5)					
RARg_TRANS	4	Imazalil (2.2)		HSE_CIS	22	Prallethrin (33)	Geldanamycin (5.7)				
RORE_CIS	35	Tetraconazole (3.3)		ISRE_CIS	0	Oxasulfuron (1.6)					
RORb_TRANS	1	Tebufenozide (2.2)		MRE_CIS	62	d-cis,trans-Allethrin (28)					
RORg_TRANS	1	Azoxystrobin (2.9)		Myb_CIS	0	Hexaconazole (1.6)					
VDRE_CIS	134	Pyridaben (5.5)		Myc_CIS	4	d-cis,trans-Allethrin (3.6)					
VDR_TRANS	0	Rotenone (1.9)		NFI_CIS	4	Prallethrin (2.6)					
PBREM_CIS	24	Prodiamine (2.8)		NF_kB_CIS	10	Dichloran (3.4)					
CAR_TRANS	4	Phosalone (2.4)		NRF1_CIS	0	Prohexadione-calcium (1.4)					
				Oct_MLP_CIS	86	Prallethrin (12)					
				p53_CIS	2	Dichlorvos (2.4)					
				Pax6_CIS	14	Prallethrin (5.2)					
				Sox_CIS	2	Cacodylic acid (2)					
				Sp1_CIS	8	d-cis,trans-Allethrin (2.9)					
				SREBP_CIS	6	d-cis,trans-Allethrin (2.5)					
				STAT3_CIS	0	Dichloran (1.8)					
				TA_CIS	4	Tribufos (2.5)					
				TAL_CIS	1	Prallethrin (2.3)					
				TCF_b_cat_CIS	0	Diphenylamine (1.7)					
				TGFb_CIS	8	Oxamyl (9.6)					
				Xbp1_CIS	12	Prallethrin (9.7)					

[a] Maximal efficacy values (*Emax*) are provided in parentheses for each listed test or positive control chemical.

triplicates were performed for the 657 chemical−assay combinations with 58 total hits (Figure S-2, Supporting Information). Including both positive and negative combinations, the overall concordance was >99%. Of the 58 hits, there was 87% concordance between the triplicates with 27 pairs agreeing and 4 disagreeing. This is equivalent to nine assay−triplicate combinations in complete agreement and two assay−triplicate combinations having only a single hit. It should be noted that any disagreement in the triplicates would be accounted for twice because every comparison is performed pairwise. Therefore, 87% hit concordance was considered high and permitted the aggregation of replicates for specific downstream analyses, making the total number of chemicals 309. For each triplicate, the average AC50 was used when all or the majority were in agreement; otherwise, the results were considered negative. The overall concordance among the duplicates was >96%, but a conservative approach was used because of the independent sourcing of the chemicals requiring both to be a hit, and the average AC50 was then used; otherwise, the results were considered negative. The complete data set is being made publicly available (Table S-2, Supporting Information) (*4*).

***In Vitro* Relationships.** Using *Emax* values, unsupervised hierarchal clustering of the 48 CIS and 25 TRANS assays demonstrated consistency across the chemical library (Figure 2). This was evidenced by coclustering of the independently tested CIS and TRANS assays corresponding to the same transcription factor function, including pregnane X receptor (PXR) with the PXR response element (PXRE), estrogen receptor alpha (ERα) with the estrogen response element (ERE), and peroxisome proliferator-activated receptor alpha and gamma (PPARα/γ) with the PPAR response element (PPRE). Such a response strongly suggests that the assay system is capable of detecting and reporting chemical perturbation of the cell, which is reflected in specific gene regulatory network alterations. Additional coherence in the data set can be observed by the chemical-induced clustering of highly related nuclear receptors across their respective subtypes, including the retinoic acid receptors (RARα, RARβ, and RARγ) and liver X receptor (LXRα and LXRβ). The corresponding response elements in the CIS assays, DR5 for RAR and DR4 for LXR, coclustered with the TRANS assays as expected. However, of the 10 NR families tested in both CIS and TRANS formats, 5 showed little evidence of coclustering primarily due to a lack of significant activity in one of the assay systems, including farnesoid X receptor (FXR), glucocorticoid receptor (GR), vitamin D receptor (VDR), constitutive androstane receptor (CAR), and RAR-related orphan receptor (ROR). A lack of activity was not unexpected for certain targets with few known ligands, including FXR, VDR, and GR, whereas CAR and ROR are constituitively active, permitting the evaluation of antagonists but not agonists. All 5 NR families with robust activity were specifically and significantly correlated across assays.

PXR/PXRE. The xenobiotic sensor, PXR, which regulates a diverse set of xenobiotic response genes including phase I, II, and III metabolic enzymes (*19*), responded to a large number of the chemicals in the library in both the CIS and TRANS format (Figure 3). While the TRANS assay used an exogenously expressed GAL4-PXR for activation of the reporter gene, the CIS system used a 628 base-pair fragment (−7836 to −7208) of a PXR-regulated gene, CYP3A4, previously identified as containing a PXRE (*20*) together with endogenous PXR. These assays showed a strong correlation with an R^2 of 0.67. As stated in the Materials and Methods section, the 95th and 99th R^2 percentile was 0.27 and 0.51, respectively, thus demonstrating the significant correlation between the independently tested PXR_TRANS and PXRE_CIS assays. However, less robust responses were observed in the TRANS version across chemicals when compared to those of the CIS assay. Responses averaged approximately 8-fold lower in the TRANS assays. Sensitivity was thus lower in the TRANS version, resulting in the derived AC50 values of the TRANS assay being a nearly complete subset of the hits in the CIS assay. In addition, ∼90% of the AC50s for TRANS hits fell within 3-fold of the PXRE_CIS AC50 (R^2 of 0.56). This is evident among the top five most efficacious and most potent PXR agonists within the chemical library (Figure 3B and C). The qualitative and quantitative similarities between the PXRE_CIS and PXR_TRANS concentration−response curves provide redundant and corroborating evidence of PXR agonism. The positive control and known human PXR ligand, rifampicin, was only run in the CIS format and showed comparable efficacy and potency with a number of the tested chemicals representative of a diverse set of chemical classes, demonstrating the promiscuous nature of PXR and the suitability of the assay for detecting such agonists.

RAR/DR5. The endogenous ligands for the retinoid acid receptors, retinoids, control key components of development, differentiation, and homeostasis (*21*). The TRANS version of reporter for the retinoid receptor assays demonstrated increased sensitivity in comparison to its response element, DR5, as shown with RARα in Figure 4A. While the reason for this is not apparent, it may be due to insufficient endogenous RAR levels in the HepG2 cells. Similar differences in sensitivity across other nuclear receptor families from CIS and TRANS versions may also be due to insufficient endogenous receptor in HepG2 cells, which is required in the CIS assays. In general, RARα, RARβ, and RARγ all showed activation by similar chemicals and were positively and significantly correlated to their response element, DR5, with R^2 values of 0.58, 0.36, 0.27, respectively. *trans*-Retinoic acid, an endogenous RAR ligand, is teratogenic when developing animals are exposed experimentally. Hence, it is important to understand both the potency and efficacy of the chemicals identified as RAR activators. The top five most efficacious and potent RAR agonists, on the basis of average RARα and DR5 results, were substantially less responsive than retinoic acid with respect to both potency and maximum efficacy (Figure 4B and C). The retinoic acid used as a positive control, tested only in the DR5_CIS assay, generated an *Emax* of 7.5 and an AC50 of 0.047 μM compared to the highest *Emax* of 3.2 and the lowest AC50 of 0.76 μM across the chemical library.

ER/ERE. Direct activation of the estrogen receptor (ER) is one mechanism for xenobiotic endocrine disruption through alteration of the physiological function of endogenous steroid hormone receptors (*22*). The TRANS ERα assay provides a measurement of receptor activation as does the ER response element (ERE), although the latter is less direct. The correlation between CIS and TRANS assays is above the 99th percentile with an R^2 of 0.53 (Figure 5). Among the most potent and/or efficacious chemicals, several are well-known estrogen receptor agonists including bisphenol A, methoxychlor, and its metabolite, HPTE (*23*−*25*). HPTE was the most potent in the chemical library but showed low efficacy most likely due to limitations on the highest concentration tested coming out of the cytotoxicity prescreen. Other chemicals showing significant activity in either the TRANS or CIS format have not been reported to be ERα agonists. Additionally, the ER TRANS assay generated 90 hits, higher than would be expected, demonstrating sensitivity but potentially lacking specificity. However, where there is good agreement across all assay types for novel findings, e.g.,

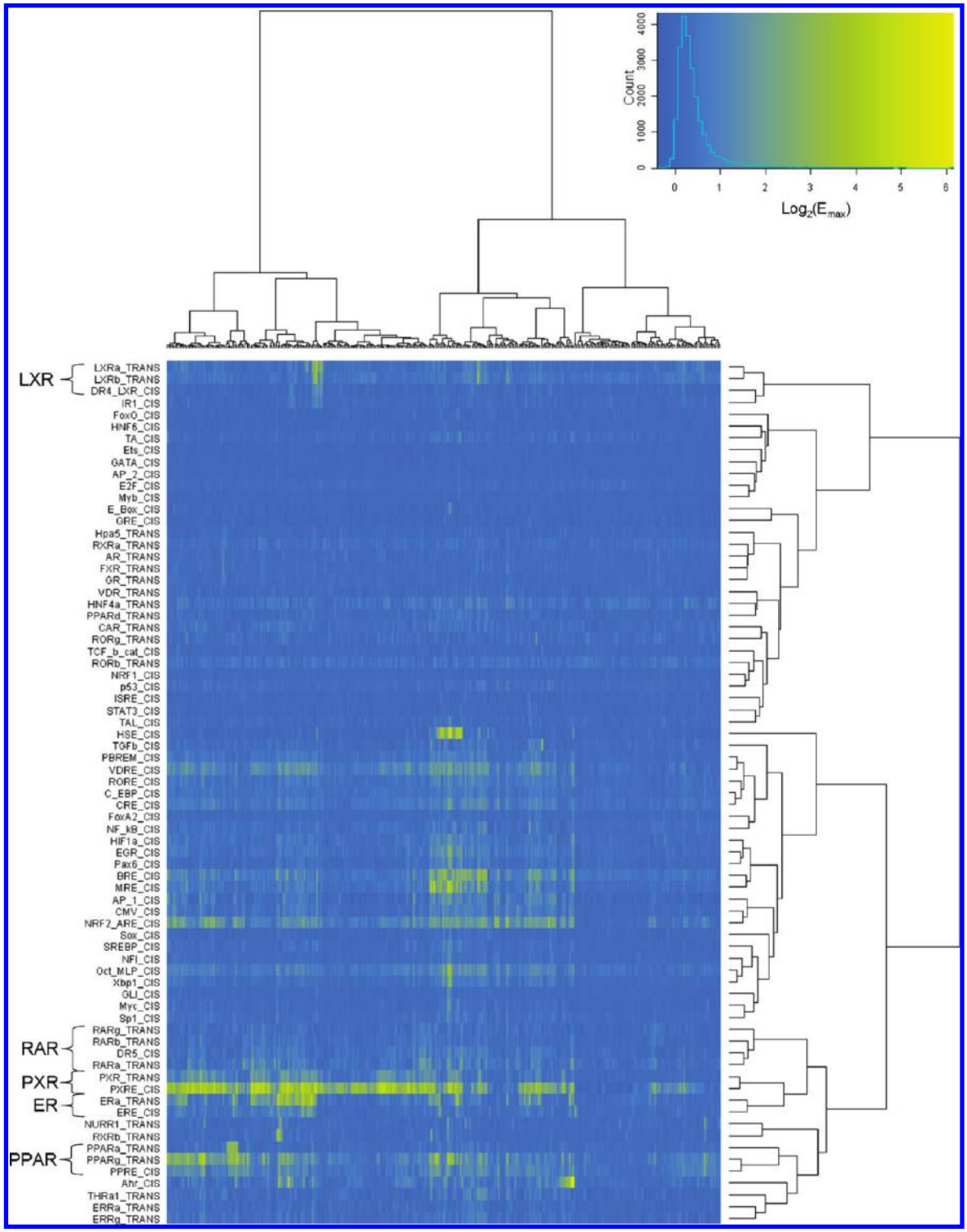

Figure 2. Two-way hierarchical clustering of the 48 CIS and 25 TRANS assays demonstrating the relationship between families of nuclear receptors and their response elements, independently tested in either the CIS or TRANS format.

fludioxonil and flumetralin, strong consideration should be given to additional testing in other assay formats to confirm the activity of these chemicals as ER ligands. It should be pointed out that many of these chemicals primarily behave as partial agonists relative to a strong endogenous ligand such as 17-β-estradiol (ERE_CIS, AC50 of 0.035 nM and *Emax* of 9.3; ERα_TRANS, AC50 of 0.87 nM and *Emax* of 23), and this may impact their biological effects both quantitatively and qualitatively.

PPAR/PPRE. The PPRE binds the PPAR subfamily (α, β/δ, and γ) to regulate genes associated with lipid metabolism (PPARα), fatty acid oxidation (PPARβ/δ), and adipocyte differentiation (PPARγ). In Figure 2, PPRE_CIS clusters with PPARα_TRANS and PPARγ_TRANS assays but not with PPARβ/δ_TRANS. The lack of significant activators of PPAR β/δ explains why it failed to cluster with the PPRE. In Figure 6A, the correlation of the PPRE and PPARγ is shown (R^2 = 0.53) with a subset of the chemicals (n = 7) also displaying PPARα activity. These apparent PPARα/γ coagonists and their involvement in rodent liver tumor formation is further investigated below. Rosiglitazone, a selective ligand of PPARγ used as the positive control, had roughly 30% greater efficacy and was 5-fold more potent than the rest of the chemicals, with the

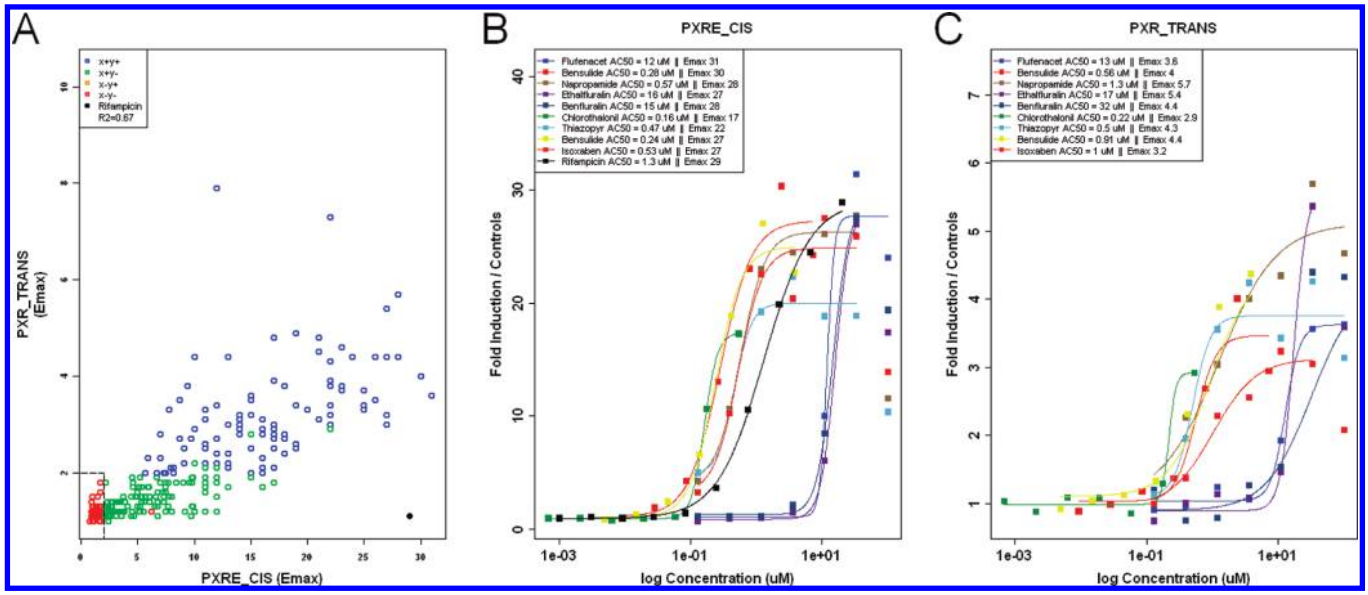

Figure 3. Comparison of pregnane X receptor (PXR) activity in the CIS (PXRE) and TRANS format characterizes both relative efficacy and potency across the chemical library. Maximum efficacy (*Emax*) plotted for both the CIS and TRANS assays across the chemical library (A). The colored circles represent the chemicals positive (AC50 derived) or negative for the assays on the *x*- and *y*-axes. (B and C) The top 5 most efficacious and top 5 most potent chemicals averaged across both assays demonstrate the relative response compared to the positive control and the representative chemicals.

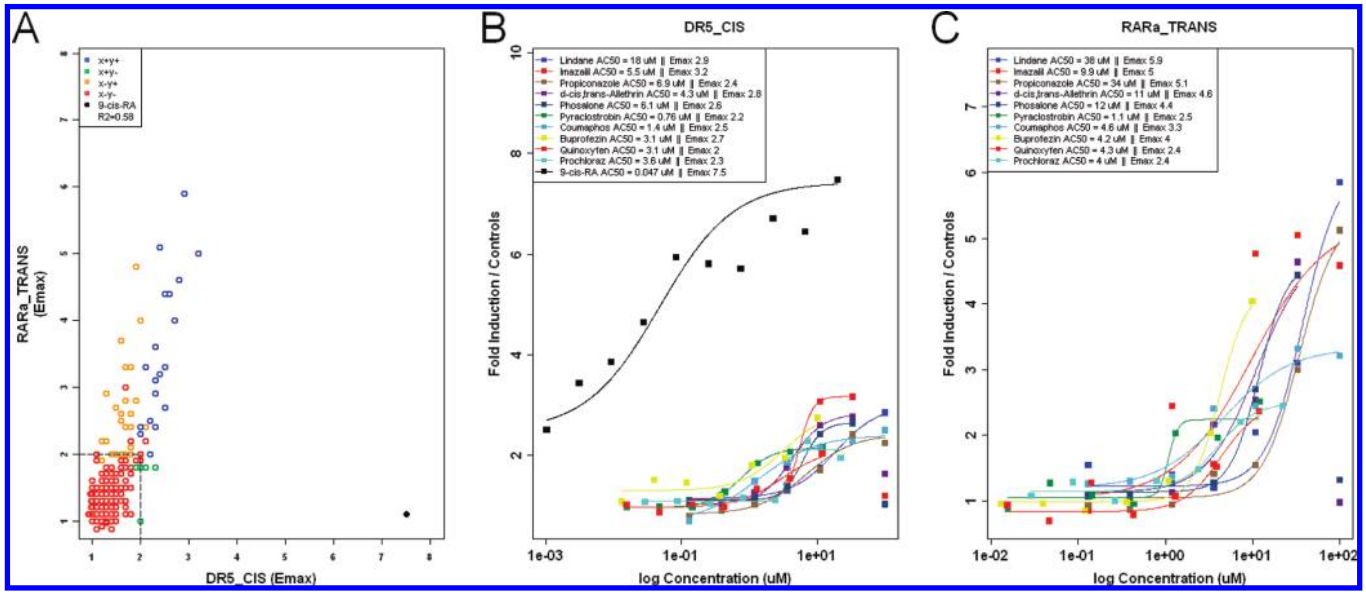

Figure 4. Comparison of retinoic acid receptor (RAR) activity in the CIS (DR5) and TRANS (RARα) format characterizes both relative efficacy and potency across the chemical library. Maximum efficacy (*Emax*) plotted for both the CIS and TRANS assays across the chemical library (A). The colored circles represent the chemicals positive (AC50 derived) or negative for the assays on the *x*- and *y*-axes. (B and C) The top 5 most efficacious and top 5 most potent averaged across both assays demonstrate the relative response compared to the positive control and the representative chemicals.

single exception of fentin in the PPRE_CIS assay (Figure 6B and C). However, fentin demonstrated substantially depressed induction in PPARγ_TRANS and no activity in PPARα_TRANS. The PPRE_CIS response may be indicative of activation of the PPAR/RXR heterodimer signal-transduction pathway through RXR agonist activity (*26*). This is supported by fentin's strong RXR responses in the RXRβ_TRANS assay with an AC50 of 83 nM. This behavior, termed permissive heterodimeric activity (*27*), has been reported for other receptor pairs in the mammalian one-hybrid assay (*28*). Rosiglitazone showed moderate induction of the PPARα_TRANS assay with an *Emax* of 3.6 and an AC50 of 6 μM reflecting partial agonist behavior at much higher concentrations than for PPARγ (data not shown), consistent with previously reported results (*29*). Similar to the situation with ERα, the biological significance of the relatively lower efficacy and potency of these chemicals compared to those of the positive control may impact high-dose animal toxicity as well as create additional uncertainty for implications for human disease.

NRF2/ARE. The nuclear-factor-E2-related factor (NRF)-2, a member of the bZIP transcription factor family, regulates cytoprotective enzymes in response to oxidants and electrophilic compounds through binding to the antioxidant response element (ARE) (*30*). Regulated genes include γ-glutamylcysteine synthetase, NADPH/quinone reductase, and glutathione-*S*-transferase (*31*). Many of the chemicals evaluated here produced significant induction of the NRF2/ARE reporter gene (Figure 7). Among the top ten activators are many pesticides known to cause oxidative damage in a variety of species. These include metolachlor (*32*), diquat dibromide (*33*), trichlorfon (*34*), oxyfluorfen (*35*), alachlor (*36*), and dichlorvos (*37*). Other

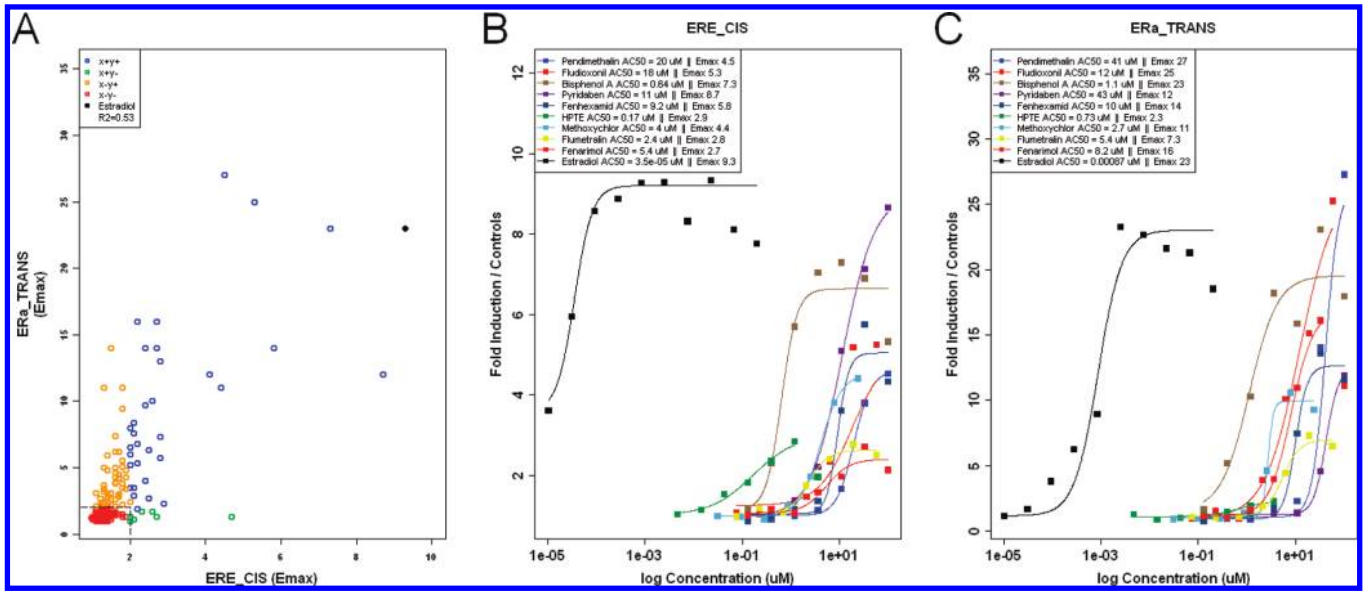

Figure 5. Comparison of estrogen receptor (ER) activity in the CIS (ERE) and TRANS (ERα) format characterizes both relative efficacy and potency across the chemical library. Maximum efficacy (*Emax*) plotted for both the CIS and TRANS assays across the chemical library (A). The colored circles represent the chemicals positive (AC50 derived) or negative for the assays on the *x*- and *y*-axes. (B and C) The top 5 most efficacious and top 5 most potent averaged across both assays demonstrate the relative response compared to the positive control and the representative chemicals.

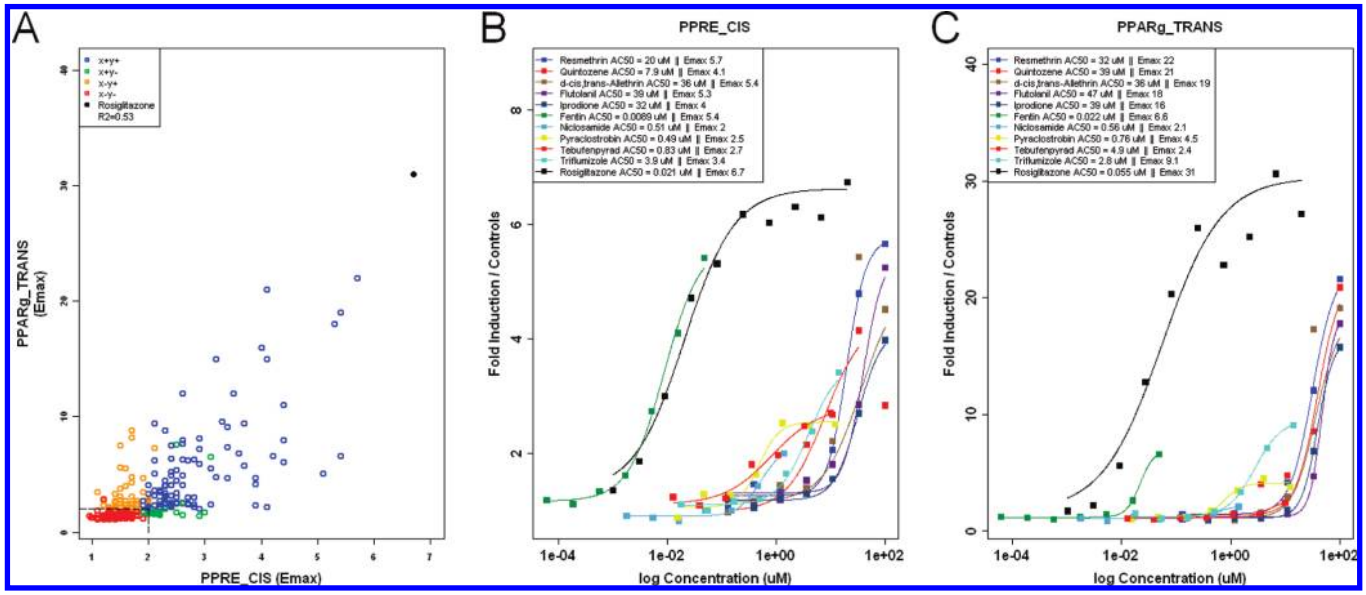

Figure 6. Comparison of peroxisome proliferator-activated receptor (PPAR) activity in the CIS (PPRE) and TRANS (PPARγ) format characterizes both relative efficacy and potency across the chemical library. Maximum efficacy (*Emax*) plotted for both the CIS and TRANS assays across the chemical library (A). The colored circles represent the chemicals positive (AC50 derived) or negative for the assays on the *x*- and *y*-axes. (B and C) The top 5 most efficacious and top 5 most potent averaged across both assays demonstrate the relative response compared to that of the positive control and the representative chemicals.

significant activators of NRF2 were compounds capable of reaction with sulfhydryl groups, e.g., methyl isocyanate (*Emax* = 3.5), a breakdown product of metam sodium (*Emax* = 6.0), dazomet (*Emax* = 3.6), and metiram-zinc (*Emax* = 3.2). As this is an adaptive response, the toxicological relevance may be very dependent on specific *in vivo* exposure scenarios. However, using the NRF2_ARE_CIS assay results as an indicator of oxidative or electrophilic damage, we observed a relationship between NRF2 response and the nonspecific and promiscuous behavior of various chemicals and assays. In comparing the NRF2 activity and the average activity across all other assays, a significant relationship emerged ($R^2 = 0.5$) (Figure 7). It should be noted that these values were log 2-transformed in order to mitigate the impacts of averaging across assays with significant differences in dynamic range. This relationship cannot be attributed to assay conditions because the positive control chemicals demonstrated very specific activity for their respective target with no evidence of NRF2 activity at comparable concentrations.

The phenomena of oxidative stress leading to promiscuous transcription factor activity may help explain the large number of active compounds in assays with fairly specific ligand binding domains, including ERα and PPARγ. For example, bisphenol A, a known ERα agonist, achieved an AC50 in 13 of the 73 assays, but only the AC50s for ERα_TRANS and ERE_CIS were significantly (>3-fold) more potent than the NRF2_ARE_CIS. Specifically, bisphenol A had AC50s of 1.1 and 0.64 μM in the ERα_TRANS and ERE_CIS assays, respectively, compared to an AC50 of 27 μM in the NRF2_ARE_CIS assay. An additional 26 chemicals hit the two ER assays and the Nrf2

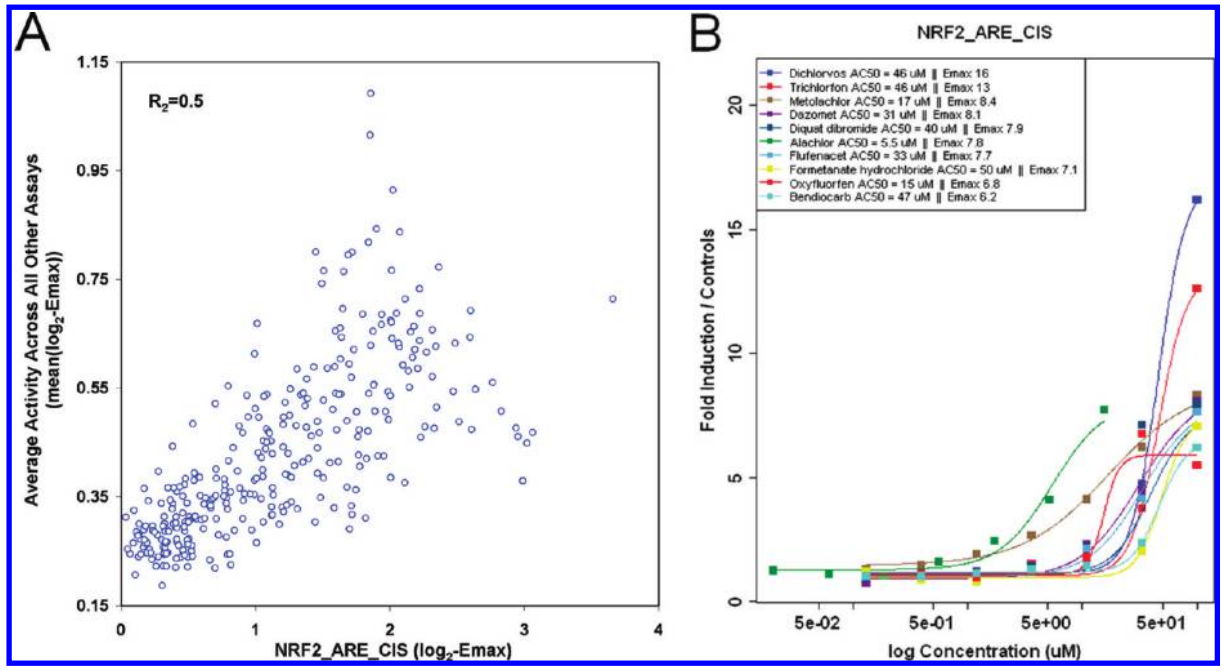

Figure 7. Oxidative stress is a plausible explanation for nonspecific activity of some chemicals. (A) Nrf2, a marker of oxidative stress, explains 50% of the variability in the overall activity across the remaining 72 targets probed in this study, both CIS and TRANS, based on the log-transformed maximal efficacy values (*Emax*). *Emax* values were log-transformed to minimize the effect of varying dynamic ranges across the assays. (B) Representative dose−response curves of the 10 most efficacious chemicals demonstrate the relative dose−response across selected chemicals.

assay, but their ERα_TRANS or ERE_CIS AC50s were all comparable to their respective NRF2_ARE_CIS AC50s. In general, these 26 chemicals showed promiscuous activity across both CIS and TRANS systems, averaging double the number of hits compared to the rest of the chemical library (11 versus 5.5). Thus, oxidative/electrophilic stress appears to elevate transcription factor activity nonselectively, and comparing potencies to NRF2 activity is therefore useful for putting compound activity into perspective and aiding the interpretation of results.

***In Vitro* to *In Vivo* Associations.** In comparing the 73 *in vitro* assay data to 77 *in vivo* end points from rodent chronic bioassays, rat multigenerational reproductive toxicity studies, and rat and rabbit prenatal developmental toxicity studies, 133 significant univariate associations were established on the basis of the methods described above (Table S-3, Supporting Information). Various nonsteroidal nuclear receptors involved in xenobiotic metabolism and/or lipid metabolism are specifically associated with target-organ pathologies and tumorigenesis, whereas steroidal and other nuclear receptors are specifically associated with endocrine-related organ pathologies, offspring survival, and the developing system (Figure 8). Examples of these associations are detailed below.

PPAR and Rat Liver Tumors. Of the 309 chemicals, 256 have two-year rat cancer bioassays recorded in ToxRefDB. Of these, 101 were found to cause tumors at one or more target sites. Of those 101 chemicals, 23 were found to cause liver tumors, specifically hepatocellular adenomas or carcinomas. These 23 chemicals were evaluated for genotoxicity throughout the pesticide registration and reregistration process and generally found to be negative (*38*), which suggests that these chemicals predominantly act through nongenotoxic mechanisms of action. In comparing the *in vitro* assay data to *in vivo* end points, only PPARα and PPARγ were significantly associated with rat liver tumors. The PPAR mode of action has been a focus of toxicological research with special emphasis on assessing relevance to humans (*39*). Additionally, the prevalence of rodent liver and other tumors in chronic toxicity studies of PPARα agonists and PPARα/γ coagonists has resulted in the U.S. Food and Drug Administration (FDA) issuing guidance for the development and use of PPAR agonists that requires special consideration be given to the carcinogenic potential of these agents (*40*). In total, 143 chemicals activated PPARγ with seven of those chemicals also activating PPARα (Figure S-3, Supporting Information). Five out of these seven chemicals (diethylhexyl phthalate (DEHP), perfluorooctanoic acid (PFOA), imazalil, lactofen, and diclofop-methyl) are positive for rat liver tumorigenicity, while only bromoxynil and fenthion are not. PPARα assay results demonstrated the ability to independently classify chemicals as potential liver tumorigens. If a chemical was considered a hit for PPARα, the relative risk for rat liver tumor induction is 9.9 ($p < 0.01$) with very high specificity (99%) and lower sensitivity. Additionally, PPARγ agonist chemicals were significantly associated with rat liver tumorigens with a relative risk of 6.6 ($p < 0.01$) but with high sensitivity (83%) and lower specificity. One could postulate that these chemicals act predominantly through a nongenotoxic peroxisome proliferation-mediated mode of action, and for a few chemicals, there is data in the literature to support this, including PFOA (*41*), DEHP (*42*), Lactofen (*43*), and Diclofop-methyl (*39*). It should be noted that oxadiazon and perfluorooctane sulfonic acid (PFOS) are purported to cause liver tumors via a PPARα mode-of-action (*44*−*46*) but were not positive under the conditions of this assay, although both of these chemicals were PPARγ agonists. PFOS was tested at a top concentration of 14 μM and oxadiazon at 24.5 μM because of the cytotoxicity observed in the MTC determination. Two reports evaluating the *in vitro* activation of human PPARα by PFOS differed with one determining an AC50 of 13−15 μM (*47*) and the other reporting no significant activity up to 250 μM (*48*); therefore, the interpretation of the present results are difficult. For oxadiazon, direct activation of PPARα has not been reported in the literature, although *in vivo* peroxisome proliferation was seen in rodents treated with high doses (≥100 mg/kg/day for

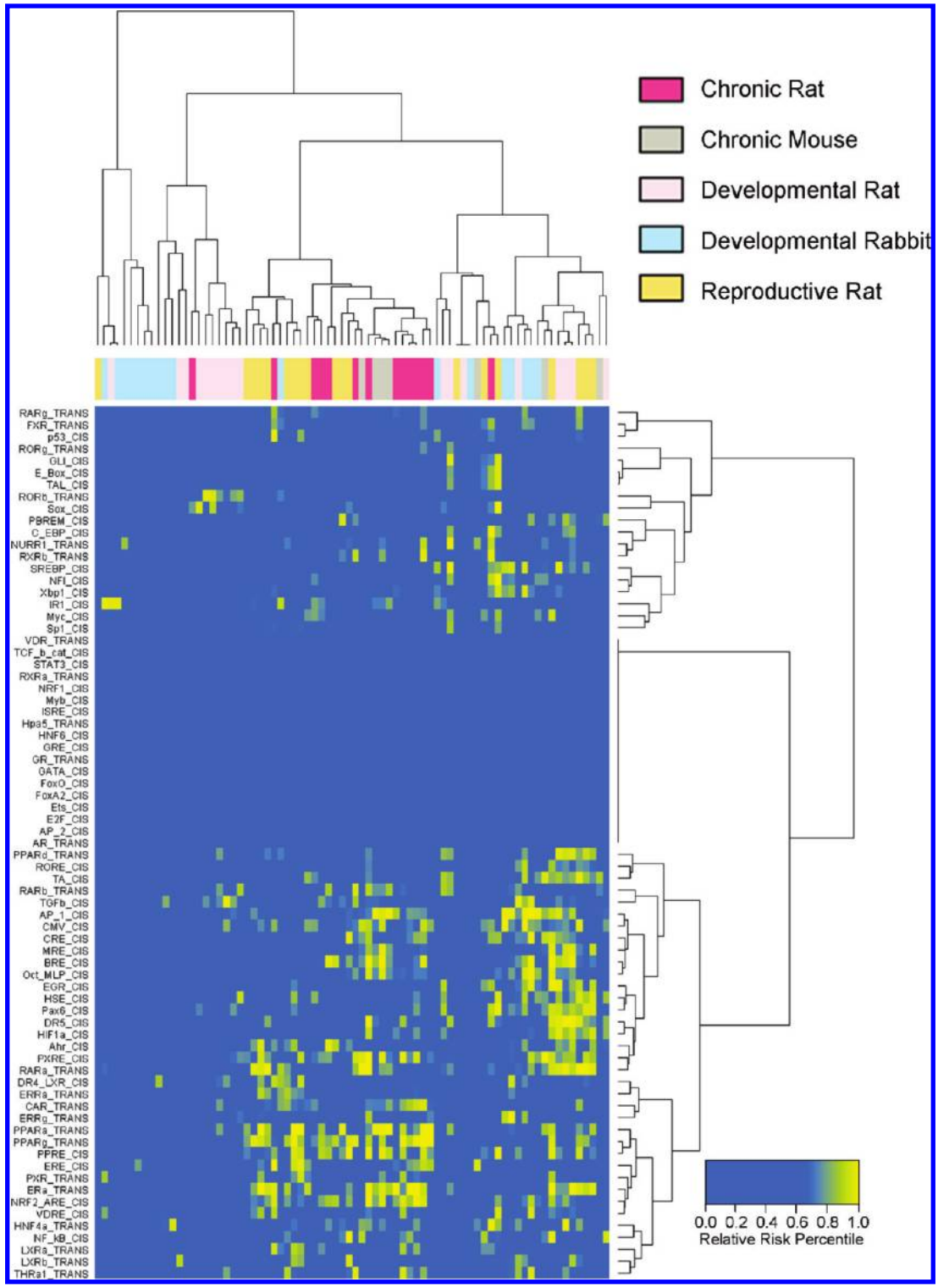

Figure 8. Two-way hierarchical clustering of the relative risk percentiles from the permutation test exhibits clustering of developmental and reproductive end points across species and study types, and grouping of systemic end points across chronic and multigeneration studies. Of the 73 *in vitro* and 77 *in vivo* end points, 133 significant ($p < 0.05$) assay/end point associations were established.

28 days), and significant peroxisomal enzyme induction measured in primary rat hepatocytes cultures required 50−100 μM (*45*).

Endocrine Pathology, Offspring Survival, and the Developing System. In total, 251 and 261 chemicals have rat multigenerational reproductive and prenatal developmental toxicity studies recorded into ToxRefDB, respectively, with 225 chemicals having both studies in ToxRefDB. Two groups of assays showed significant associations with specific reproductive and developmental processes or targets (Table 2). As shown above, RARα and DR5 were correlated to each other, and RAR has direct biological links to the developing system (*21*). Chemicals positive for RARα/DR5 have a significantly greater potential for effects in various developmental systems and at different stages of development, including orofacial defects (i.e., cleft lip/palate) and urogenital malformations (i.e., renal and ureteric). In contrast, the estrogen receptor alpha (ERα) and its response element (ERE), a known endrocrine disrupting target, were associated with reproductive effects, including reproductive performance (i.e., decreased fertility and implantation loss). In

Table 2. Two Distinct Molecular Targets with Known Links to Developmental and Reproductive Toxicities, Retinoic Acid Receptor and Estrogen Receptor, Demonstrate Coherent Associations to *in Vivo* Developmental and Reproductive End Points from ToxRefDB and Are Shown with Their Respective Significant Relative Risk (RR) Values ($p < 0.05$)

	prenatal developmental			multigeneration reproductive						
	malformations			reproductive performance			offspring survival			
assay	cleft lip/palate	renal	urogenital	fertility	gestational interval	implantations	litter size	live birth index	viability index	lactation index
ERa_TRANS			5			9	2	2		
ERE_CIS				3						
RARa_TRANS	4	3			4	4	2	2	2	2
DR5_CIS	5	5	6							

addition to their specific associations, RARα/DR5 and ERα/ERE appear to have similar impacts on early offspring survival as indicated by shared associations to litter size and live birth outcomes. Interestingly, RARα/DR5 and ERα/ERE activity is not correlated with an R^2 of roughly zero, meaning there shared associations are independent and may reflect varying mechanisms leading to the same adverse outcome. In general, the predictive power of probing these molecular targets for tissue-specific observations such as testicular or adrenal effects is limited, whereas data on hormone or cholesterol levels would most likely provide direct links from molecular to phenotypic alterations but are not available for sufficient numbers of compounds in this chemical library.

Discussion

Cells respond to changes in their environment through a variety of recognition systems and signaling pathways that are integrated at the level of transcription factors, which coordinate appropriate responses. The multiplexed reporter gene technology described here attempts to probe this biological response by generating profiles of individual chemicals acting against a large panel of transcription factors in two different assay systems. These profiles provide information on potential molecular interactions of the chemicals, as well as more generalized activity such as induction of oxidative and electrophilic stress. Quantitation of the effects of chemicals on specific transcription factor responses provides information that can be used to determine the potential for toxicity. Characterizing the responses to known toxicants in these assays allows the magnitude and potency of responses for new chemicals to be put into an appropriate context for evaluation. Testing chemicals in both the CIS and TRANS systems revealed specific limitations of each system, as well as complementarity of the data from each system. Valuable insights for interpreting large screening data sets came from comparing CIS and TRANS data, especially when limitations of one system, e.g., the lack of endogenous activity for a particular target, were attenuated by data from the other. In cases where both complementary assays demonstrated robust responses, greater confidence resulted from associating a given chemical with a specific molecular activity.

Chemicals can both directly and indirectly affect transcription factor activities. The nuclear receptor superfamily and the Ah receptor are examples of TFs that can bind xenobiotic chemicals directly through their ligand-binding domains, resulting in the modulation of target gene activity. In some cases, receptors such as PXR appear to have evolved to recognize such xenobiotics and to control the expression of a large series of phase I, phase II, and phase III metabolizing enzymes and transporters (*49*). Whereas such interactions generally serve to help eliminate or detoxify a xenobiotic, they may also lead to undesirable consequences, resulting in a variety of toxicities when exposure is sufficiently high. For example, the induction of the phase I, II, and III activities can also cause effects on endogenous compounds, e.g., steroid hormone metabolism, altering normal physiology. In addition, such enzyme induction can cause interference with the expected metabolism of pharmaceuticals leading to side effects resulting from either too high or too low a dose of the drug (*50*). Endocrine disruption is another example, whereby xenobiotics mimic natural ligands such as steroid hormones in binding to nuclear receptors, resulting in the disruption of normal receptor signaling and potentially leading to reproductive toxicities (*51*). Outside the endocrine system, chemicals activating PPARα cause peroxisome proliferation in rodents and subsequent hepatocarcinogenesis, a cancer mode of action that does not seem to occur in humans (*39*). Thus, screening environmental chemicals for the ability to affect ligand-activated transcription factors can provide a means to recognize the potential for the chemical to cause endocrine disruption and other toxicities.

Chemical effects on cells may also be detected through effects on nonligand-activated TFs. Such TFs include those that respond to a wide variety of cellular stressors, including oxidative, electrophilic, hyperosmolarity, DNA damage, hypoxia, etc. These stress-response effects enable a cell to survive environmental changes by adapting through increasing levels of target gene products involved in reducing or buffering the stress, e.g., increasing glutathione levels to handle oxidative stress or heat-shock proteins to provide protection to newly synthesized proteins during heat or UV light stress (*31*). When stress is beyond the tolerance of the adaptive systems, cell death occurs through necrosis or apoptosis, the latter allowing selective removal of irreparably damaged cells from the tissue. Examples of effects seen in the current study included many chemicals inducing oxidative/electrophilic stress (Nrf-2/ARE CIS) but few inducing DNA damage as measured by the activation of the p53 CIS assay. The latter finding is consistent with the majority of the chemicals tested being food-use pesticides that would not be expected to have significant genotoxic effects. Because transcription factors of this general functional class are involved in adaptive responses, changes in their activation do not necessarily equate directly to toxicity. Rather, they suggest that the chemical inducing the response possesses a mode of action that may, under sufficient exposure conditions, lead to effects that can overwhelm the capacity of the adaptive response, resulting in toxicity. Evaluating the potential for the toxicity of chemicals with such activity will require careful consideration of potency and exposure to properly inform estimations of risk.

In contrast to the receptors involved in nuclear receptor signaling and stress response, many of the other transcription factors screened in this study are known to be involved with specific signaling pathways controlling growth and differentiation. For example, E2F, MYC, and SMAD regulate growth in virtually all cells (*52*), whereas OCT, PAX, SOX, GLI, and TCF are among the factors involved in specific aspects of cellular differentiation (*53*). In general, the reporter genes for these response elements showed only marginal response to the

chemical library. The reasons for this are not clear. One possibility is that the signaling pathway controlling the specific reporter gene response is not functional under the conditions used for the assay. Not every reporter gene response was evaluated with suitable positive controls, and thus, activity could only be inferred from the fact that some basal level of reporter gene product was present in the cell. Use of a different cell line may be one way to expand functional pathway coverage. In addition, by prescreening for cytotoxicity, we ensured that frankly cytotoxic concentrations of chemicals would not be used in the screen. It is possible that interference with many of the transcription factors required for growth and differentiation results in cytotoxicity, and thus, the screening strategy employed to avoid this biased the results against finding effects on these pathways. Future screening efforts will take both of these points into consideration.

Relating effects observed with *in vitro* assays using transformed cell lines to *in vivo* toxicity remains a significant challenge. Here, we evaluated statistical correlations between chemical effects on transcription factors and toxicity end points, the latter collated in a relational database derived from extensive animal testing in support of the U.S. pesticide registration process. The relatively few significant associations detected were plausible associations between peroxisome proliferator-activated receptors and liver toxicity, estrogen receptor activity correlating to reproductive toxicities, and retinoic acid receptor toxicities associated with developmental defects. These results were confirmed by the coclustering of specific targets across the CIS and TRANS systems, thus increasing confidence in the *in vivo* associations. The application of *in vitro* screening to chemical prioritization is enhanced by multiple, complementary assays such as these for the same target. There are many possible explanations for the inability to discern more correlations. The associations between these selected groups of molecular targets and apical end points do not encompass all possible routes by which a chemical can induce an adverse effect or toxic outcome. Likewise, it may be that there are insufficient chemicals within the current library representing each possible route to enable the detection of significant associations. It is also understood that there are significant uncertainties in drawing associations between high-dose laboratory animal toxicity studies and *in vitro* animal target activities, much less the further extrapolation from *in vitro* human molecular target activities, despite the greater relevance of the latter to potential human risk. Chemical *in vivo* effects in relation to *in vitro* effects also may be greatly affected by chemical ADME properties (adsorption, distribution, metabolism, and elimination), with metabolic biotransformation, in particular, resulting in either much greater or reduced toxicity than might otherwise be predicted. Whereas the HepG2 cell line used here is derived from the liver and maintains some metabolic capacity, it is much less than that in the intact liver (*54*). Finally, many of the responses seen were statistically significant; however, the magnitude of the response was frequently much less than that seen with a positive control compound. Again, relating the level of efficacy observed in the *in vitro* assay to biological meaningful results *in vivo* poses significant challenges.

The multiplexed RTU assay results evaluated in the present study encompass only a fraction of the full range of biological targets and assays, both cell-free and cell-based, being run against the ToxCast phase I chemical library (*4*). However, the present assays are based on human transcription factors and, thus, are believed to have particular potential relevance to humans and to informing *in vivo* extrapolations. The associations reported in this study, many of which are corroborated by results reported in the literature, demonstrate the biological relevance of several of the presently studied molecular targets for monitoring and probing toxicity pathways and for understanding the mechanisms of action leading to specific chemical toxicities. Along with chemical properties and ADME considerations, combining these activity profiles with additional *in vitro* assay profiles having the potential to probe a broader range of biological responses in animals and humans, will offer greater opportunities to discern meaningful associations of *in vitro* profiles with *in vivo* effects. Ongoing efforts to enlarge the test chemical library will additionally enrich the chemical and biological information dimensions in relation to toxicity end points of regulatory interest and, in so doing, provide greater coverage of toxicity mechanisms and facilitate the development of robust methods suitable for prioritizing chemicals based on the potential for toxicity.

Acknowledgment. This data was generated by Attagene under U.S. EPA Contract Number EP-W-07-049, and we thank Mr. John Southerland for excellent management of this contract. We also thank Dr. Maritja Wolf, Lockheed Martin, contractor to the U.S. EPA, for technical assistance managing chemical information. D.M.R. was supported by EPA/University of North Carolina Department of Environmental Sciences and Engineering Cooperative Training Agreement #CR83323601. Attagene research was supported by U.S. National Institutes of Health grants 1R43CA101636, 1R43CA101271, and UO1AI061360. The United States Environmental Protection Agency through its Office of Research and Development funded and managed the research described here. It has been subjected to Agency administrative review and approved for submission and peer review.

Supporting Information Available: The 48 CIS and 25 TRANS assay names and descriptions, the complete concentration response and derived AC50 values, and the results of the relative risk permutation test as tables, and figures displaying the results of establishing a global *Emax* cutoff, the replicate analysis, and the association between PPAR and rat liver tumorigenesis. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Wilson, V. S., Bobseine, K., and Gray, L. E. (2004) Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. *Toxicol. Sci. 81*, 69–77.
(2) Houck, K. H., and Kavlock, R. J. (2008) Understanding mechanisms of toxicity: Insights from drug discovery research. *Toxicol. Appl. Pharmicol. 227*, 163–178.
(3) NRC. (2007) *National Research Council, Toxicity Testing in the 21st Century: A Vision and a Strategy*, National Academy Press, Washington DC.
(4) U.S. EPA. (2009) EPA ToxCast. http://www.epa.gov/ncct/toxcast/ (accessed Aug 8, 2009).
(5) Collins, F. S., Gray, G. M., and Bucher, J. R. (2008) Transforming environmental health protection. *Science 319*, 906–907.
(6) Dix, D. J., Houck, K. A., Martin, M. T., Richard, A. M., Setzer, R. W., and Kavlock, R. J. (2007) The ToxCast program for prioritizing toxicity testing of environmental chemicals. *Toxicol. Sci. 95*, 5–12.
(7) Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., and Makarov, S. S. (2008) Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. *Nat. Methods 5* (3), 253–60.
(8) U.S. EPA. (2009) EPA DSSTox TOXCST: Research Chemical Inventory for EPA's ToxCastTM Program: Structure-Index File, http://www.epa.gov/ncct/dsstox/sdf_toxcst.html (accessed 8 August 2009).
(9) Houck, K. A., Dix, D. J., Judson, R. S., Kavlock, R. J., Yang, J., and Berg, E. L. (2009) Profiling bioactivity of the ToxCast chemical library

using BioMAP primary human cell systems. *J. Biomol. Screening 14* (9), 1054−1066.
(10) Martin, M. T., Judson, R. S., Reif, D. M., Kavlock, R. J., and Dix, D. J. (2009) Profiling chemicals based on chronic toxicity results from the U.S. EPA ToxRef database. *Environ. Health Perspect. 117*, 392–399.
(11) Martin, M. T., Mendez, E., Corum, D. G., Judson, R. S., Kavlock, R. J., Rotroff, D. M., and Dix, D. J. (2009) Profiling the reproductive toxicity of chemicals from multigeneration studies in the toxicity reference database (ToxRefDB). *Toxicol. Sci. 110*, 181–190.
(12) Knudsen, T. B., Martin, M. T., Kavlock, R. J., Judson, R. J., Dix, D. J., and Singh, A. V. (2009) Profiling the activity of environmental chemicals in prenatal developmental toxicity studies using the U. S. EPA's ToxRefDB. *Reprod. Toxicol. 28*, 209–219.
(13) U.S. EPA. (2009) EPA Toxicity Reference Database, http://www.epa.gov/NCCT/toxrefdb/ (accessed Jun 4, 2009).
(14) Medvedev, A., Moeser, M., Poltoratskaya, N., Gambarian, M., Medvedeva, L., Gambarian, M., Martsen, E., Romanov, S., and Makarov, S. S. Global functional profiling of nuclear receptor ligands, manuscript in preparation.
(15) Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods 65* (1−2), 55–63.
(16) Ihaka, R., and Gentleman, R. (1996) R: A language for data analysis and graphics. *J. Comput. Stat. 5*, 299–314.
(17) Ekins, S., Mirny, L., and Schuetz, E. G. (2002) Ligand-based approach to understanding selectivity of nuclear hormone receptors PXR, CAR, FXR, LXRalpha, and LXRbeta. *Pharmacol. Res. 19* (12), 1788–800.
(18) Lemay, D., and Hwang, D. H. (2006) Genome-wide identification of peroxisome proliferator response elements using integrated computational genomics. *J. Lipid Res. 47*, 1583–1587.
(19) Gillam, E. M. (2002) Coordinating clearance: The many phases of PXR! *Trends Pharmacol. Sci. 23*, 548–549.
(20) Goodwin, B., Hodgson, E., and Liddle, C. (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol. Pharmacol. 56* (6), 1329–39.
(21) Chambon, P. (1996) A decade of molecular biology of retinoic acid receptors. *Fed. Am. Soc. Exp. Biol. J. 10*, 940–954.
(22) Witorsch, R. J. (2000) Endocrine disruption: a critical review of environmental estrogens from a mechanistic perspective. *Toxic Subst. Mech. 19*, 53–78.
(23) Gould, J. C., Leonard, L. S., Maness, S. C., Wagner, B. L., Conner, K., Zacharewski, T., Safe, S., McDonnell, D. P., and Gaido, K. W. (1998) Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. *Mol. Cell. Endocrinol. 142* (1−2), 203–14.
(24) Gray, L. E., Jr., Ostby, J., Ferrell, J., Rehnberg, G., Linder, R., Cooper, R., Goldman, J., Slott, V., and Laskey, J. (1989) A dose-response analysis of methoxychlor-induced alterations of reproductive development and function in the rat. *Fundam. Appl. Toxicol. 12*, 92–108.
(25) Gaido, K. W., Maness, S. C., McDonnell, D. P., Dehal, S. S., Kupfer, D., and Safe, S. (2000) Interaction of methoxychlor and related compounds with estrogen receptor alpha and beta, and androgen receptor: structure-activity studies. *Mol. Pharmacol. 58* (4), 852–8.
(26) le Maire, A., Grimaldi, M., Roecklin, D., Dagnino, S., Vivat-Hannah, V., Balaguer, P., and Bourguet, W. (2009) Activation of RXR-PPAR heterodimers by organotin environmental endocrine disruptors. *EMBO Rep. 10* (4), 367–73.
(27) Aranda, A., and Pascual, A. (2001) Nuclear hormone receptors and gene expression. *Physiol. Rev. 81* (3), 1270–1295.
(28) Bettoun, D. J., Burris, T. P., Houck, K. A., Buck II, D. W., Stayrook, K. R., Khalifa, B., Lu, J., Chin, W. W., and Nagpal, S. (2003) Retinoid X receptor is a nonsilent major contributor to vitamin D receptor-mediated transcriptional activation. *Mol. Endocrinol. 17* (11), 2320–2328.
(29) Reifel-Miller, A., Otto, K., Hawkins, E., Barr, R., Bensch, W. R., Bull, C., Dana, S., Klausing, K., Martin, J. A., Rafaeloff-Phail, R., Rafizadeh-Montrose, C., Rhodes, G., Robey, R., Rojo, I., Rungta, D., Snyder, D., Wilbur, K., Zhang, T., Zink, R., Warshawsky, A., and Brozinick, J. T. (2005) A peroxisome proliferator-activated receptor alpha/gamma dual agonist with a unique in vitro profile and potent glucose and lipid effects in rodent models of type 2 diabetes and dyslipidemia. *Mol. Endocrinol. 19* (6), 1593–605.
(30) Nguyen, T., Nioi, P., and Pickett, C. B. (2009) The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J. Biol. Chem. 284* (20), 13291–13295.
(31) Lee, J. M., and Johnson, J. A. (2004)) An important role of Nrf2-ARE pathway in the cellular defense mechanism. *J. Biochem. Mol. Biol. 37* (2), 139–43.
(32) Stajner, D., Mimico-Dukic, N., Milosevic, M., and Zlokolica, M. (2001) Herbicide stress and its effect on scavenging system of vegetable sorts. *Oxid. Commun. 24*, 602–608.
(33) Wolfgang, G. H. I., Jolly, R. A., Donarski, W. J., and Petry, T. W. (1991) Inhibition of diquat-induced lipid peroxidation and toxicity in precision-cut rat liver slices by novel antioxidants. *Toxicol. Appl. Pharmacol. 108*, 321–329.
(34) Karademir, A., Durmusoglu, E., and Bakoglu, M. (2007) Health risk assessment of background PCDD/F exposure levels in Kocaeli, Turkey. *J. Environ. Sci. Health, Part A: Toxic/Hazard. Subst. Environ. Eng. 42* (6), 729–39.
(35) Peixoto, F., Alves-Fernandes, D., Santos, D., and Fontainhas-Fenandes, A. (2006) Toxicological effects of oxyfluorfen on oxidative stress enzymes in tilapia Oreochromis niloticus. *Pestic. Biochem. Physiol. 85*, 91–96.
(36) Burman, D. M., Shertzer, H. G., Senft, A. P., Dalton, T. P., and Genter, M. B. (2003) Antioxidant perturbations in the olfactory mucosa of alachlor-treated rats. *Biochem. Pharmacol. 66* (9), 1707–15.
(37) Yarsan, E., and Cakir, O. (2006) Effects of dichlorvos on lipid peroxidation in mice on subacute and subchronic periods. *Pestic. Biochem. Physiol. 86* (2), 106–109.
(38) U.S. EPA. (2009) Pesticide Reregistration Status, http://www.epa.gov/oppsrrd1/reregistration/status.htm (accessed Jun 4, 2009).
(39) Lai, D. Y. (2004) Rodent carcinogenicity of peroxisome proliferators and issues on human relevance. *J. Environ. Sci. Health, Part C: Environ. Carcinog. Ecotoxicol. Rev. 22* (1), 37–55.
(40) U.S. FDA. (2008) Guidance for Industry, Diabetes Mellitus: Developing Drugs and Therapeutic Biologics for Treatment and Prevention, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm071624.pdf (accessed Aug 11, 2009).
(41) Lau, C., Butenhoff, J. L., and Rogers, J. M. (2004) The developmental toxicity of perfluoroalkyl acids and their derivatives. *Toxicol. Appl. Pharmacol. 198* (2), 231–241.
(42) Melnick, R. L. (2001) Is peroxisome proliferation an obligatory precursor step in the carcinogenicity of di(2-ethylhexyl)phthalate (DEHP)? *Environ. Health Perspect. 109* (5), 437–442.
(43) Butler, E. G., Tanaka, T., Ichida, T., Maruyama, H., Leber, A. P., and Williams, G. M. (1988) Induction of hepatic peroxisome proliferation in mice by lactofen, a diphenyl ether herbicide. *Toxicol. Appl. Pharmacol. 93* (1), 72–80.
(44) DeWitt, J. C., Shnyra, A., Badr, M. Z., Loveless, S. E., Hoban, D., Frame, S. R., et al. (2009) Immunotoxicity of perfluorooctanoic acid and perfluorooctane sulfonate and the role of peroxisome proliferator-activated receptor alpha. *Crit. Rev. Toxicol. 39* (1), 76–94.
(45) Richert, L., Price, S., Chesne, C., Maita, K., and Carmichael, N. (1996) Comparison of the induction of hepatic peroxisome proliferation by the herbicide oxadiazon in vivo in rats, mice, and dogs and in vitro in rat and human hepatocytes. *Toxicol. Appl. Pharmacol. 141* (1), 35–43.
(46) U.S. EPA. (2003) Proposed OPPTS Science Policy: PPAR-Mediated Hepatocarcinogenesis in Rodents and Relevance to Human Health Risk Assessments, http://www.epa.gov/scipoly/SAP/meetings/2003/december9/peroxisomeproliferatorsciencepolicypaper.pdf (accessed Aug 11, 2009).
(47) Shipley, J. M., Hurst, C. H., Tanaka, S. S., DeRoos, F. L., Butenhoff, J. L., Seacat, A. M., and Waxman, D. J. (2004) Trans-activation of PPARalpha and induction of PPARalpha target genes by perfluorooctane-based chemicals. *Toxicol. Sci. 80* (1), 151–60.
(48) Takacs, M. L., and Abbott, B. D. (2007) Activation of mouse and human peroxisome proliferator-activated receptors (alpha, beta/delta, gamma) by perfluorooctanoic acid and perfluorooctane sulfonate. *Toxicol. Sci. 95* (1), 108–17.
(49) Kliewer, S. A., Goodwin, B., and Willson, T. M. (2002) The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. *Endocr. Rev. 23*, 687–702.
(50) Wilkinson, G. R. (2005) Drug metabolism and variability among patients in drug response. *New Engl. J. Med. 352*, 2211–2221.
(51) WHO. (2002) Global assessment of the state-of-the-science of endocrine disruptors, International Programme on Chemical Safety, World Health Organization, http://www.who.int/ipcs/publications/en/ch1.pdf (accessed Aug 11, 2009).
(52) Grandori, C., Cowley, S. M., James, L. P., and Eisenman, R. N. (2000) The Myc/Max/Mad network and the transcriptional control of cell behavior. *Annu. Rev. Cell Dev. Biol. 16*, 653–99.
(53) Maeda, Y., Dave, V., and Whitsett, J. A. (2007) Transcriptional control of lung morphogenesis. *Physiol Rev. 87*, 219–244.
(54) Donato, M. T., Lahoz, A., Castell, J. V., and Gómez-Lechón, M. J. (2008) Cell lines: a tool for in vitro drug metabolism studies. *Curr. Drug Metab. 9* (1), 1–11.

TX900325G