Integration of Dosimetry, Exposure, and High-Throughput Screening Data in Chemical Toxicity Assessment


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High-throughput in vitro toxicity screening can provide an efficient way to identify potential biological targets for chemicals. However, relying on nominal assay concentrations may misrepresent potential in vivo effects of these chemicals due to differences in bioavailability, clearance, and exposure. Hepatic metabolic clearance and plasma protein binding were experimentally measured for 239 ToxCast Phase I chemicals. The experimental data were used in a population-based in vitro–to-in vivo extrapolation model to estimate the dose of the human oral dose, called the oral equivalent dose, necessary to produce steady-state in vivo blood concentrations equivalent to in vitro AC50 (concentration at 50% of maximum activity) or lowest effective concentration values across more than 500 in vitro assays. The estimated steady-state oral equivalent doses associated with the in vitro assays were compared with chronic aggregate human oral exposure estimates to assess whether in vitro bioactivity would be expected at the dose-equivalent level of human exposure. A total of 18 (9.9%) chemicals for which human oral exposure estimates were available had oral equivalent doses at levels equal to or less than the highest estimated U.S. population exposures. Ranking the chemicals by nominal assay concentrations would have resulted in different chemicals being prioritized. The in vitro assay endpoints with oral equivalent doses lower than the human exposure estimates included cell growth kinetics, cytokine and cytochrome P450 expression, and cytochrome P450 inhibition. The incorporation of dosimetry and exposure provide necessary context for interpretation of in vitro toxicity screening data and are important considerations in determining chemical testing priorities.

Key Words: reverse dosimetry; in vitro–to-in vivo extrapolation; toxicokinetics; human exposure; ToxCast.

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For several decades, toxicity testing has relied on the administration of high doses of a chemical to laboratory animals to identify potential adverse effects. These testing approaches were established at a time when knowledge of mode of action and the role of signaling pathways in biological responses was limited, and the high-dose effects in laboratory species were presumed to be indicative of effects at relevant human exposures (Andersen and Krewski, 2009). Although advances in our understanding of endpoints relevant to human health risk have added additional testing requirements for specific chemicals, an overall reevaluation of both the relevance and the benefits of the current testing requirements has yet to occur (NRC, 2007). One consequence of the complexity and expense of the current testing paradigm is that, with certain exceptions such as food-use pesticidal active ingredients, relatively few chemicals in commerce have been fully evaluated for toxicity (Allanou et al., 2003; EPA, 1998; Judson et al., 2009; Wilson and Schwarzman, 2009).

To address concerns regarding the large number of relatively untested chemicals and to improve chemical risk management, the U.S. Environmental Protection Agency (EPA) has implemented the ToxCast research program to evaluate hundreds to thousands of chemicals in a broad panel of in vitro high-throughput screening (HTS) assays at a fraction of the cost and time of in vivo animal studies (Dix et al., 2007). In the first phase of the ToxCast program, a library of 309 unique chemicals, consisting primarily of food-use pesticides and high-production volume chemicals, was screened in concentration-response format across hundreds of cell-based and biochemical assays (Houck et al., 2009; Huang et al., 2011; Judson et al., 2010; Knight et al., 2009; Knudsen et al., 2011; Martin et al., 2010; Rotroff et al., 2010a). The potency of each chemical in each positive assay was summarized using AC50 (concentration at 50% of maximum activity) or lowest effective concentration (LEC) values, depending on the type of dose-response data that were collected for each
assay. The pattern of nominal potency values among the in vitro assays along with other chemical information has been proposed for use in hazard identification and in prioritizing chemicals for further testing (Reif et al., 2010). However, hazard identification and prioritization based on nominal in vitro assay concentrations without considering the impact of bioavailability, clearance, and exposure can over- or underestimate the potential risk of these chemicals to human health (Blauauber, 2010).

In this study, we evaluated the utility of integrating human dosimetry and exposure information with in vitro toxicity HTS data across the majority of the Phase I ToxCast chemicals. This investigation extends results of an earlier study where a small subset of the ToxCast Phase I chemicals were analyzed (Rotroff et al., 2010b). For human dosimetry, in vitro assays were performed for each chemical to estimate hepatic metabolic clearance and plasma protein binding. Computational in vitro–to–in vivo extrapolation (IVIVE) methods were used to calculate the daily human oral dose, called the oral equivalent dose, that would be required to produce steady-state in vivo blood concentrations of a chemical equivalent to the in vitro AC50 and LEC value from each of the previously published ToxCast assays. These oral equivalent doses for each chemical-assy combination were compared with the human oral exposure estimates (derived either following consideration of human activity [e.g., consumption of certain goods] or back calculated from excreted metabolite data) to assess whether in vitro bioactivity would be expected at the dose-equivalent level of human exposure. Our results show that incorporation of dosimetry and exposure information improve the ability to prioritize chemicals for further testing and to evaluate the potential human health effects at relevant levels of exposure.

MATERIALS AND METHODS

Biochemicals. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, gentamicin, penicillin/streptomycin, and GlutaMAX were purchased from Invitrogen Corporation (Grand Island, NY). ITS+ was purchased from BD Biosciences (San Jose, CA). William’s E Medium, dexamethasone, acetonitrile, dimethyl sulfoxide (DMSO), and Trypan Blue were obtained from Sigma Chemical Co. (St Louis, MO).

Chemical selection and stock preparation. The 239 chemicals analyzed in this study comprise a subset of the 309-chemical ToxCast Phase I chemical library (http://www.epa.gov/ncct/toxcast/chemicals.html) for which analytical detection methods were readily available. Data for 35 of these 239 chemicals were analyzed and discussed previously (Rotroff et al., 2010b) and were included in this paper to provide a comprehensive assessment of incorporating dosimetry as it applies to the more complete ToxCast Phase I chemical inventory. The complete ToxCast Phase I chemical library consisted of 309 unique compounds selected largely on the availability of extensive in vivo toxicity data but also filtered by solubility in DMSO, molecular weight, and purity (Houck et al., 2009). Compounds for the plasma protein and metabolic stability assays were obtained either commercially or from Compound Focus, Inc., a subsidiary of BioFocus DPI (South San Francisco, CA) in neat form or as 20mM stock solutions in DMSO supplied in 96-well polypropylene plates (Supplementary table 1). Solutions were prepared from the neat chemicals to generate the analytical calibration curves. All stock solutions were stored at < −70°C. Neat chemicals were stored either at < −70°C (those supplied in 96-well format) or as directed by the vendor. Of the 239 chemicals analyzed, 16 (6.7%) had a documented purity < 95%. Of these, two had a documented purity < 90%—chlorothoxyfos (60.0%) and iodosulfuron-methyl-sodium (89.0%). Three chemicals had no purity information available (cinmethinyl, endosulfan, and fentin hydroxide). Specific vendor and vendor-supplied purity information for each chemical is provided as supplementary material (Supplementary table 1).

Plasma protein binding assay. Plasma protein binding was determined for each chemical using the rapid equilibrium dialysis (RED) method with slight modification (Rotroff et al., 2010b; Waters et al., 2008). Human plasma was obtained using anti-coagulant (K2-EDTA) from healthy consented paid donors at a U.S. Food and Drug Administration-licensed and inspected donor center (#HMPLEDTA2, Bioreclamation, Inc., Westbury, NY). The plasma was pooled from three males (34, 23, and 46 years old) and three females (56, 35, and 21 years old) donors and tested negative for HBSAg, HIV 1/2 Ab, HIV-1 RNA, HCV Ab, HCV RNA, and STS. The plasma (stored at < −70°C until use) was thawed at room temperature and centrifuged at 2000 × g for 10 min prior to analysis. The RED assay was conducted in 96-well format using single-use RED plates (catalog no. 90006, Pierce Biotechnology, Rockford, IL) according to manufacturer’s instructions. Chemical stock solutions (prepared in DMSO) were added to the plasma chambers to achieve a final concentration of 10μM. The concentration of DMSO did not exceed 0.5%. The RED plate was wrapped in aluminum foil and incubated at 37°C at 100 oscillations per minute for 4 h. After incubation, aliquots were removed and equal volumes of PBS or plasma were added to aliquots from the plasma or PBS chambers, respectively, for matrix matching. The samples were diluted with three times the volume of 100% acetonitrile and stored at < −70°C until analytical analysis. The RED assays were performed in triplicate.

Metabolic clearance assay. The rate of hepatic metabolism of the parent compound was determined as previously described (Rotroff et al., 2010b) with slight modification. Chemicals at two concentrations (1 and 10μM) were incubated over a 4-h period with cryopreserved primary human hepatocytes (Gibco Life Technologies, Corp.; Durham, NC). The 1μM concentration is a standard concentration used in metabolic stability assays in the pharmaceutical industry (Naritomi et al., 2001; Obach, 1999). In addition, both concentrations fall in the middle of the range of concentrations tested in the ToxCast assays. The cryopreserved hepatocytes were obtained from two separate pools of ten individual donors (HuP50 for the initial 35 compound study [Rotroff et al., 2010b], and HuP2000 for the balance of the Phase 1 ToxCast chemicals). Both pools were made from five male and five female donors. The HuP50 pool was made from nine Caucasian donors and one African American donor. The HuP2000 pool was made from eight Caucasian donors, one African American donor, and one Hispanic donor. Both pools of hepatocytes were characterized for metabolism (CYP1A2, CYP2C9, CYP2D6, CYP3A4/5, CYP2C19, ethoxyco- marin glucuronidation, and ethoxyco- marin sulfation) and viability (Trypan Blue exclusion), and the values from each lot fell within acceptable ranges compared with historical quality control limits (Supplementary table 2). The human hepatocytes were obtained under a protocol that was reviewed and approved by an Institutional Review Board that operated in accordance with Federal Regulation for the protection of human research subjects.

Chemical stock solutions (prepared at 0.2 and 2mM in DMSO) were added to prewarmed (37°C) incubation medium (William’s E Medium containing 0.1μM dexamethasone, 4mM GlutaMAX 15mM HEPES, ITS+ supplement, and 2 μg/ml gentamicin or 2.5 μl penicillin/streptomycin per 500 ml medium) in polystyrene tubes to achieve 2× working stock chemical concentrations of 2 and 20μM using a Multimek Liquid Handler (Beckman Coulter, Brea, CA). These solutions were then transferred to 96-well polypropylene plates (0.05 ml per well) and incubated at 37°C and 5% CO2 for 10–30 min prior to addition of the cells.
Vials of cryopreserved hepatocytes were thawed, transferred to 50-ml vials containing the CHRM (Gibco, Life Technologies) hepatocyte thaw medium, centrifuged at 76 × g for 6 min at 20°C, then cell pellets were resuspended in incubation medium (William’s E Medium) to a working density of 1.0 × 10^6 viable cells/ml as determined by Trypan Blue exclusion. Cells were “gently” (wide bore tips, 0% speed adjustment) added to the incubation plates using a liquid handler (Multimek) to final cell densities of 0.5 × 10^6 viable cells/ml and final chemical concentrations of 1 and 10μM (DMSO concentration did not exceed 0.5%). Plates were shaken at 300 oscillations per minute in the incubator until removed at their respective time points (0, 15, 30, 60, 120, and 240 min). Plate contents were then quenched with ice-cold acetonitrile (100 μl), transferred to polypropylene matrix tubes, and stored at −8°C until analysis. A media-only (no cell) negative control and a negative matrix control (boiled hepatocytes) were included for each chemical. Each sample was run in triplicate.

**Bidirectional permeability (Caco-2) assay.** To assess the impact of bioavailability on the IVIVE of the ToxCast chemical library, a subset of the chemicals were tested in the bidirectional permeability (Caco-2) assay. Caco-2 cells (passage numbers 65–66; 21–28 days old) were grown to confluency in Hank’s balanced salt solution (HBSS) on polycarbonate Transwell inserts. Chemical stock solutions prepared at 10mM in DMSO were administered in duplicate to the apical (for apical to basolateral assessment, A → B) or basolateral (for basolateral to apical assessment, B → A) side at pH 7.4 ± 0.2 to achieve a final concentration of 5μM (DMSO concentration not to exceed 0.8%). Media was collected from the receiver and donor wells 120 min after chemical addition. The receiver wells contained 1% bovine serum albumin in HBSS to minimize nonspecific binding of chemical to the plasticware. Percent recovery and the apparent permeability (P_{app}) in both directions (apical to basolateral [P_{app} A-B] and basolateral to apical [P_{app} B-A]) were determined. The quality and integrity of the monolayer batch were assessed by measurement of transepithelial electrical resistance and by determining the P_{app} for control compounds propanolol, atenolol, and lucifer yellow. The permeability assays were performed at Absorption Systems LP (Exton, PA).

**Red blood cell partitioning assay.** To assess the impact of red blood cell partitioning on the IVIVE of the ToxCast chemical library, a subset of the chemicals were tested in this assay. Reference plasma was isolated from an aliquot of fresh whole human blood treated with K$_2$EDTA following centrifugation at 700 × g for 15 min. Chemical stock solutions prepared at 50mM in DMSO were administered to both whole blood and reference plasma to achieve a final concentration of 5μM (DMSO concentration not to exceed 0.5%) and incubated with shaking for 60 min at 37°C. Whole blood was then centrifuged at 700 × g for 15 min while plasma was stored on ice prior to addition of acetonitrile. Samples were shaken at RT for 5 min and then centrifuged at 6000 × g at 4°C for 15 min prior to measurement of the compound in the supernatant. Samples were run in duplicate. The K_{RCBCPL} was determined using the following equation:

$$K_{RCBCPL} = \frac{(I_{REF/PL}/I_{PL}) - 1}{H} + 1,$$

where H, hematocrit; I REF/PL, area ratio of reference plasma spiked with compound at same concentration as that of the whole blood; and I PL = the area ratio of the equilibrating plasma from the whole blood spiked with compound for testing. Quality of the assay was assessed through the concurrent analysis of reference compound verapamil.

**Chemical analysis by high performance liquid chromatography with mass spectrometric detection.** Samples from the metabolic stability assay (quenched 1:1 with acetonitrile) were thawed at room temperature, vortexed briefly, and centrifuged at 12,000 × g for 5 min. All plasma samples were prepared as outlined above for the 1μM metabolic stability assay samples (i.e., 1:4 dilution).

Chromatographic separation was conducted on a C$_{18}$ column (Allure C$_{18}$, 50 × 2.1 mm, 3.0 μm) (Restek, Bellefonte, PA) with a C$_{18}$ guard column. Aqueous mobile phases used for these analyses were either 0.1% FA, for positive ionization, or 10mM ammonium acetate (pH 6.8), for negative ionization, and methanol for the organic mobile phase. Samples (50-μl injections) were analyzed using a solvent gradient consisting of the following steps: (1) 20% organic for 0.5 min; (2) linear gradient ramp to 100% organic over 4.5 min; (3) maintain 100% organic for 1 min; (4) linear gradient ramp to 20% organic over 0.5 min; and (5) maintain 20% organic for 2 min prior to the next injection. Total analysis time was 8.5 min per sample. The flow rate used for this analysis was 200 μl per minute and was introduced into the mass spectrometer in splitless mode. Mass spectrometry conditions for all compounds are described in Supplementary table 3.

**Chemical analysis by selective ion-monitoring gas chromatography with mass spectrometric detection.** Both metabolic stability assay samples and protein binding samples were obtained in the same dilutions described in the high performance liquid chromatography with mass spectrometric (HPLC/MS) methods above. All samples were thawed at room temperature, vortexed briefly, and centrifuged at 12,000 × g for 5 min. Prior to solid phase extraction (SPE), samples were diluted 1:10 with an aqueous solution containing a known amount of internal standard (Parathon, CAS no. 56-38-2). Samples were applied to previously conditioned 96-well C$_{18}$ SPE plates (cat. no. 186003966; Waters, Milford, MA). Diluted samples (1.0 ml total volume) were pulled through the SPE columns using a vacuum manifold, washed once with 500 μl of deionized water per well, and eluted using 500 μl of HPLC grade methanol per well. Methanol eluents were collected and transferred to silanized glass inserts prior to analysis using an Agilent 6890 GC with model 5973 MS (Agilent Technologies, Santa Clara, CA) in either electron impact ionization mode or negative chemical ionization mode. Calibration standards were constructed and prepared on the same day as sample analysis and in a matrix identical to the samples. Sample data was collected in selective ion monitoring mode. Specific instrumental parameters for each analyte are provided as supplementary material (Supplementary table 4).

Chromatographic separation was conducted on 1 μl sample injections using a Restek Rtx-5MS column (30 m, 0.25 mm ID, 0.25 μm film thickness) (Restek) in splitless mode. The temperature gradient used in the analysis consisted of the following steps: (1) initial injection port temperature was 275°C with an initial oven temperature of 125°C; (2) oven temperature maintained for 0.5 min; (3) oven temperature increased at 30°C per min to 300°C; and (4) oven temperature maintained at 300°C for 2.7 min. Total analysis time was 9.0 min.

**Chemical analysis by HPLC with UV/Vis detection.** Samples from both the metabolic stability assay and the protein binding assay were thawed at room temperature and briefly vortexed prior to centrifugation at 12,000 × g for 5 min. Samples were placed in silanized glass inserts and injected onto an Agilent 1100 HPLC with UV/Vis detector (Agilent Technologies) without any additional sample work-up. Chromatographic separation was conducted on 50 μl sample injections using a C$_{18}$ column (Allure C$_{18}$, 250 × 4.6 mm, 5.0 μm) (Restek) with a C$_{8}$ guard column. The mobile phases used for this analysis were 10mM ammonium acetate (pH 6.8) in water and methanol. Calibration standards were prepared on the same day as sample analysis and in a matrix...
identical to the samples. The gradient conditions used for this analysis were as follows: (1) initial conditions consisted of 25% organic for 2 min, (2) linear gradient ramp to 100% organic over the next 10 min, (3) maintain 100% organic for 7 min, (4) linear gradient to 25% organic over the next 1.5 min, and (5) equilibrate column at 25% organic for 1.5 min prior to analysis of the next sample. Flow rate for the analysis was 1.0 ml/min for a total of 20 min. Samples were analyzed with a column temperature of 30°C and a UV/Vis absorbance wavelength of 258 nm. Analyte elution times are provided in the supplementary materials section (Supplementary table 5).

**Plasma protein binding data analysis.** To calculate percent of unbound chemical (Fub), the mean concentration of the test compound in the PBS chamber (n = 3 replicates) was divided by the mean concentration in the matched plasma sample (n = 3 replicates) and multiplied by 100. A minimum measurable Fub was set to 0.005. This value was estimated based on two standard deviations over the minimum amount of binding detected in a previous study (Waters et al., 2008) and on practical experience with the RED method. If the concentration of the chemical in the free fraction was below this value or below the analytical limits of detection, a default Fub of 0.005 was assumed.

**Metabolic clearance data analysis.** Metabolic clearance data were plotted separately in semilog format (log concentration vs. time) with three replicates at each time point. The disappearance of the chemical over time was analyzed using linear regression. The concentration data at each time point for each chemical are provided as Supplementary table 6. Clearance was normalized to cell number. Considering three replicates at each of the six time points, a standard F-test (degrees of freedom = 1, 16; α = 0.10) was used to determine whether the slope of the line was significantly different from 0. For data sets with measurements that fell below detection before the 4-h time point, the degrees of freedom were adjusted accordingly. For chemicals that fell below detection levels before the 4-h time point and were not statistically significant (p > 0.10), values were interpolated to determine whether the lack of statistical significance was influenced by data falling below detection limits. For a small subset of chemicals, liquid-handling errors resulted in a single time point falling below the detection limit. For these chemicals, the time point was removed from the analysis and the degrees of freedom adjusted accordingly. Chemicals that had no statistically significant change (p > 0.10) were assigned a metabolic clearance of 0. Analysis of the data from Rotroff et al. (2010b) was similar but varied based on the use of two replicates and five time points in the assay (degrees of freedom = 1, 8; α = 0.10).

**In vitro bioactivity data.** The initial phase of the Toxicast program measured activity of 390 compounds against a set of approximately 500 in vitro assays. Nine separate technologies were used, including receptor-binding and enzyme activity assays, cell-based protein and RNA expression assays, real-time growth measured by electronic impedance, and fluorescent cellular imaging. Each chemical-assy combination was run in dose response and an AC50 or LEC value was estimated depending on the range of the dose response data. The in vitro bioactivity was assumed to be solely the result of the parent compound. Although two assays used primary hepatocyte cultures with some metabolic capacity, most of the assays lacked known metabolic activity. A detailed description of the assays and associated data are provided in earlier publications (Houck et al., 2009; Huang et al., 2011; Judson et al., 2010; Knight et al., 2009; Knudsen et al., 2011; Martin et al., 2010; Rotroff et al., 2010a). All data are available from the Toxicast web site (http://www.epa.gov/nct/toxicast).

**Estimation of oral equivalents using IVIVE.** A simple pharmacokinetic equation (Wilkinson and Shand, 1975) was used to estimate expected steady-state blood concentrations. The equation was based on zero-order uptake of a daily dose from the gut (assuming 100% oral bioavailability) with both renal and hepatic clearance. The steady-state concentration in the blood is

\[ C_{ss} = \frac{\text{mo}}{(\text{GFR} \times F_{ub}) + (Ql \times F_{ub} \times C_{int}/(Ql + F_{ub} \times C_{int}))}, \]

where ko, input rate (mg/kg/h); Fub, unbound fraction of parent compound in the plasma; GFR, glomerular filtration rate; Ql, liver blood flow; and Cint, intrinsic metabolic clearance for first-order conditions of metabolism in liver. For the glomerular filtration rate, a value of 111 ml/min/1.73 m² or 6.7 l/h was used (Rule et al., 2004). For the metabolic clearance, either the 1 or 10µM value was used depending on which value was closer to the AC50 or LEC concentration. For several chemicals, the metabolic clearance was only successfully measured at one of the two test concentrations. In this case, the measured clearance was used in the Cint determination.

Simulations were performed using a dose of 1 mg/kg/day and the Simcyp software (Simcyp Limited, Sheffield, U.K.). Monte Carlo analysis was performed within the Simcyp software (Jamei et al., 2009) to simulate variability across a population of 100 healthy individuals of both sexes from 20 to 50 years of age. A coefficient of variation of 30% was used for intrinsic and renal clearance. The median, upper, and lower fifth percentiles for the concentration at steady state (Cint) were obtained as output.

In conventional use, pharmacokinetic models are used to relate exposure concentrations to a blood or tissue concentration. This is typically referred to as “forward dosimetry.” In contrast, the models can also be reversed to relate blood or tissue concentrations to an exposure concentration, which is referred to as “reverse dosimetry.” Based on the principal of reverse dosimetry, the median, upper, and lower fifth percentiles for the concentration at steady state were used as conversion factors to generate oral equivalent doses according to the following formula:

\[ \text{Oral equivalent dose (mg/kg/day)} = \frac{\text{ToxCast AC50 or LEC (µM)}}{C_{int} (µM)} \times \frac{1}{\text{mg/kg/day}} \]

In the equation above, the oral equivalent value is linearly related to the in vitro AC50 or LEC and inversely related to Cint. This equation is valid only for first-order metabolism that is expected at ambient exposure levels. An oral equivalent value was generated for each chemical and each AC50 or LEC value across the 500 in vitro assays. These chemical and assay combinations that did not show activity (i.e., did not possess an AC50 or LEC value) were not simulated.

**Statistical presentation of oral equivalents data.** The oral equivalent doses derived from the upper 95th percentile Cint values were used to provide a conservative estimate in the analyses. The oral equivalent doses determined for each chemical-assay combination are presented as box-and-whisker plots for each chemical with the median displayed as a horizontal line and the ends of the boxes representing the 25th and 75th percentiles. The whiskers denote those values that fall either less than or greater than 1.5 times the interquartile range from the 25th and 75th percentile values, respectively (Tukey, 1977). In those instances where the lowest or highest value for that chemical-assay combination does not exceed the whisker, the whisker is set to that value. Any value beyond the range of the whiskers is designated as an outlier and is displayed as a black circle.

**Evaluation of pharmacokinetic modeling.** Published studies of the human in vivo pharmacokinetics of 2,4-dichlorophenoxyacetic acid (Kohli et al., 1974; Sauerhoff et al., 1977), bisphenol A (Völkell et al., 2002), and acetylsalicylic acid (Brown et al., 1997; Buecher et al., 1981; El-Masri and Kenyon, 2008), carbaryl (May et al., 1992), fenitrothion (Mecklin et al., 2003), lindane (Dick et al., 1997), oxytetracycline dihydrate (Green et al., 1976), parathion (Gentry et al., 2002), perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) (Loccisano et al., 2010), picloram (Nolan et al., 1984), thiabendazole (Baprio et al., 2005), and triclosan (Sandborgh-Englund et al., 2006) were used to estimate the Cint in the plasma in humans exposed to 1 mg/kg/day.

**Estimation of human oral exposure.** For most of the chemicals, exposure estimates were obtained from available EPA Office of Pesticide Programs documents and Federal Register notices. The majority of the estimates came from Reregistration Eligibility Documents that contained residue levels and estimated aggregate exposures from food and drinking water sources organized by various age groups and subpopulations. For four of the chemicals—bisphenol A, diethylhexyl phthalate, dimethyl phthalate, and mono-n-butyl phthalate—exposures were estimated using the urinary concentrations provided in the U.S. Centers for Disease Control and Prevention’s (CDC) Fourth
National Report on Human Exposure to Environmental Chemicals (NHANES) (CDC, 2010). Of the various demographics sampled by NHANES (e.g., children 6–11 years, males, Mexican Americans), the demographic with the highest 95% value (µg/l urine) for the chemical in question was selected to provide a conservative estimate of exposure. For the selected demographic, urine outputs (l/day) were determined based on LaKind and colleagues (LaKind and Naiman, 2008; LaKind et al., 2008) and body weights (kg) were based on CDC’s National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population 1999–2002 (CDC, 2008). These values were then used to determine the mg/kg/day exposure for each chemical, assuming that individuals were at steady state. For those chemicals with multiple metabolites (e.g., diethylhexyl phthalate and dimethyl phthalate), the metabolite concentrations have also been incorporated into the parent compound estimate. Whereas the use of metabolite concentrations to infer parent concentration is inexact because other metabolites may have been missed and other compounds could produce these metabolites, this information was incorporated to provide a more conservative exposure estimate. For two of the chemicals—triclosan and cacodylic acid—exposure estimates were determined from both Reregistration Eligibility Documents and NHANES data. For both, the estimate provided by the former was the most conservative (by 6.1- and 11.2-fold greater, respectively) and was used as the exposure estimate in our analyses. The exposure estimates are provided in Supplementary table 7.

RESULTS

Plasma Protein Binding Assay

Measurement of plasma protein binding revealed that most of the chemicals were highly bound to plasma, with 59% of the chemicals having a fraction unbound 0–5% (Fig. 1A). Only 7.1% of the chemicals tested had fraction unbound values greater than 80%, whereas no unbound chemical was detected for 77 of the chemicals (32%).

Hepatic Metabolic Clearance Assay

The metabolic clearance measurements ranged from 0 to 250.30 and 0 to 234.42 l/min/10^6 cells at the 1 and 10 µM concentrations, respectively. Of these chemicals, 82 (34%) showed either no metabolism or saturation at the 10 µM concentration and 37 (15%) showed either no metabolism or saturation at both the 1 and 10µM concentrations. Conversely, 202 of the 239 (85%) chemicals displayed clearance in at least one of the two concentrations. Most (78%) of the chemicals had no metabolism or had clearance values < 20 µl/min/10^6 cells (Fig. 1B).

IVIVE Modeling

Of the 239 chemicals analyzed in this study, published pharmacokinetic information—from which steady-state concentrations (C_{ss}) values could be determined—was available for 13. The C_{ss} values based on the published models were compared against those derived using a population-based IVIVE model across a cohort of 100 healthy individuals where median C_{ss} values were predicted assuming a dose rate of 1 mg/kg/day and using the 1µM metabolic clearance rate (Table 1). For comparison purposes, two alternative hepatic clearance assumptions (Wilkinson and Shand, 1975) were employed: (1) restrictive hepatic clearance using F_{ub} determined experimentally in the plasma protein binding assay and (2) nonrestrictive hepatic clearance where the F_{ub} was set to 0.99. In both cases, the renal clearance was based on the experimentally derived F_{ub} and the GFR. Using the restrictive hepatic clearance assumption, the IVIVE model predictions for six chemicals (2,4-dichlorophenoxyacetic acid, bisphenol A, cacodylic acid, carbaryl, oxytetracycline dihydrate, and triclosan) were comparable to the C_{ss} values derived from published models. Of the seven remaining chemicals, the IVIVE model significantly (i.e., by greater than an order of magnitude) overpredicted the C_{ss} values for five chemicals (fenitrothion, lindane, parathion, picloram, and thiabendazole) and underpredicted for two chemicals (PFOS and PFOA). Incorporation of the permeability assay data into the IVIVE model as a measure of bioavailability increased the predictivity of the C_{ss} determination for two of the 13 chemicals assessed: oxytetracycline dihydrate and picloram. Incorporation of blood partitioning assay data into the model had no effect on the C_{ss} values of the chemicals (data not shown). Using the nonrestrictive

FIG. 1. Distributions of the in vitro pharmacokinetic assay data for the 239 ToxCast Phase I chemicals analyzed. Histograms of the (A) percentage of unbound data from the plasma protein binding measurements and (B) hepatic clearance measurements (includes both the 1 and 10µM concentrations).
hepatic clearance assumption, the IVIVE model predictions for eight of the 13 chemicals (2,4-dichlorophenoxyacetic acid, bisphenol A, cacodylic acid, carbaryl, fenitrothion, oxytetracycline dihydrate, parathion, and picloram) were comparable to the published studies, with four of the remaining chemicals underpredicted (lindane, PFOS, PFOA, and triclosan) and one overpredicted (thiabendazole). The trend toward overprediction of \( C_{ss} \) under the assumption of restrictive hepatic clearance would lead to a lower and more conservative estimate of the oral equivalent values. As a result, the model with restrictive hepatic clearance was used in the broader assessment of \( C_{ss} \) values across the 239 chemicals.

It should be noted that published \emph{in vivo} pharmacokinetic information does exist for dimethoate (Brown et al., 1997; Hoffmann and Papendorf, 2006; Tarbah et al., 2007). However, the data were derived from dimethoate poisoning cases and due to reported renal toxicity of dimethoate (Mahjoubi-Samet et al., 2008), the \( C_{ss} \) value could not be accurately estimated, and data were not used in the validation comparison.

Due to the inverse relationship between the \( C_{ss} \) and the oral equivalent dose, the upper 95th percentile of the \( C_{ss} \) was used to obtain a conservative estimate of the lower fifth percentile oral equivalent dose for each chemical-assay combination with a measurable AC\(_{50}\) or LEC value (Fig. 2). The lower fifth percentile oral equivalent dose ranges for each chemical are summarized as box-and-whisker plots (Figs. 3A–D). Oral equivalent doses for each chemical-assay combination that exceed the whiskers are designated as outliers and are represented by solid black circles. Of the chemicals tested, fentin hydroxide had the lowest oral equivalent dose range, with a median of 5.00 ng/kg/day and a minimum of 0.03 ng/kg/day. Diazoxon had the highest oral equivalent dose range, with a median of 564.9 mg/kg/day. Importantly, the number of \emph{in vitro} ToxCast assays used to generate the box-and-whisker plots varied with the number of measurable AC\(_{50}\) or LEC values and ranged from 1 assay for mesosulfuron-methyl to 139 assays for emamectin benzoate. The complete results from the IVIVE computational modeling as well as the plasma protein binding values, metabolic stability values, and the assay AC\(_{50}\) or LEC values for each chemical are provided in Supplementary table 8.

**Estimated Human Oral Exposures**

Chronic aggregate human oral exposure estimates were obtained for 182 of the 239 chemicals (76%). For most chemicals, oral exposures were estimated for multiple age and gender-based subpopulations. A comparison of the exposure estimates with the oral equivalent doses (Figs. 3A–D) revealed that 18 chemicals (9.9%) possessed human exposure estimates for the most highly exposed subpopulation that overlapped with the range of nonoutlier oral equivalent doses. These chemicals were 2-phenylphenol, acifluorfen, chlorpropham, cyprodinil, dicamba, dichloran, fenbuconazole, fenhexamid, fludioxonil, fluoroxypry-methyl, isoxaben, piperonyl butoxide, prometon, pyraclostrobin, quinclorac, spiroxamine, and...
tetraconazole, and triclosan. Additional chemicals—bensulfuron-methyl, ethalfluralin and quinoxyfen—had oral equivalent dose ranges that were only slightly higher than their respective human exposure estimates. When compared against exposure estimates for the general U.S. population, 10 of the 18 chemicals had overlapping oral equivalent doses (5.5% of the 182).

In vitro assay endpoints for which the predicted oral equivalents were lower than the upper estimate of human exposure are listed in Table 2. Supplementary table 9 contains Table 2 information along with the specific assay names. Assay endpoints include prostaglandin E receptor (PTGER2) downregulation, urokinase-type plasminogen activator (PLAU) downregulation, decreased cell growth kinetics, upregulated expression of sulfotransferase 2A1 (SULT2A1) and solute carrier organic anion transporter family member 1B1 (SLCO1B1), and enzymatic inhibition and change in expression of several cytochrome P450 (CYP450) isozymes. The number of assays per chemical that yielded oral equivalent values below the estimated human exposure ranged from 1 to 7 (Table 2).

To more broadly characterize the differences between the oral equivalent dose ranges and the human exposure estimates, activity-to-exposure ratios (AERs) were calculated for each of the ToxCast Phase I chemicals using in vitro assays. The pharmacokinetic data were used to parameterize an IVIVE model. Monte Carlo simulation using the IVIVE model provided estimates of the variability of the steady-state blood concentration ($C_{ss}$) in a population of healthy individuals of both sexes from 20 to 50 years old. Using reverse dosimetry, oral doses were then estimated that would result in a steady-state blood concentration equivalent to the AC$_{50}$ or LEC value in each of the ToxCast assays. The range of the oral equivalent doses required to achieve the upper 95th percentile $C_{ss}$ across all the in vitro ToxCast assays was represented as a box plot.

**DISCUSSION**

The report by the National Research Council “Toxicity Testing in the 21st Century” (NRC, 2007) has spawned multiple research efforts in the United States and Europe aimed toward use of in vitro HTS assays in the toxicological assessment of environmental chemicals (Abbott, 2009; Kavlock et al., 2009; Knight, 2008). However, meaningful incorporation of these in vitro findings into such an assessment is dependent upon adequate consideration of in vivo pharmacokinetics to determine the relevance of these data to the external and internal doses achieved during human exposure scenarios (Blaauboer, 2010; Hays and Aylward, 2009). Within the pharmaceutical industry, IVIVE modeling approaches have been widely used to assess the preclinical pharmacokinetics of candidate molecules (Caldwell et al., 2009; De Buck and Mackie, 2007). In the environmental field, pharmacokinetic
FIG. 3. Comparison of human oral equivalent dose ranges and oral exposure estimates for the ToxCast Phase I chemicals analyzed. The distribution of the oral equivalent dose ranges required to achieve the upper 95th percentile $C_{ss}$ across all the in vitro ToxCast assays for each chemical is depicted as a box-and-whisker plot. The 239 chemicals are displayed across four panels (A–D), ordered from lowest to highest median oral equivalent dose. Horizontal lines depict the medians, the lower and upper edges of the boxes represent the 25th and 75th percentiles, and the whiskers represent the range of values 1.5 times the interquartile range below or above the 25th and 75th percentiles, respectively. In those instances where the lowest or highest value for that chemical-assay combination does not exceed the whisker, the whisker is set to that value. Human oral exposure estimates were obtained for 182 of the 239 chemicals analyzed. The orange floating
boxes represent the range of exposure estimates obtained for various age- and gender-based subpopulations. The green circles represent the exposure estimates for the general U.S. population. Exposure estimates for some of the chemicals fell below the units on the axes and are therefore not shown on the graphs. Chemicals where any of the exposure estimates fall within the range of predicted oral equivalents are highlighted with arrows.
models have been used to estimate chemical concentrations in human biofluids, consistent with reference dose (RfD), or other exposure guidance values used to interpret biomonitoring data (Hays et al., 2008; LaKind et al., 2008). By combining advances in the pharmaceutical and biomonitoring fields, focused in vitro pharmacokinetic assays and IVIVE modeling offer a promising approach for the interpretation of in vitro toxicity HTS data through the estimation of oral equivalent doses expected to produce blood concentrations in exposed humans equivalent to in vitro concentrations showing activity in our various HTS assays.

In this study, in vitro assays were performed on all the ToxCast Phase I chemicals for which analytical detection methods were available to estimate two critical determinants of pharmacokinetics—hepatic metabolic clearance and plasma protein binding. Eighty-five percent (202 of the 239 chemicals analyzed in this study) showed significant metabolic clearance in at least one of the two concentrations tested. The range (0–250 μl/min/10^6 cells), distribution and median values obtained for these environmental chemicals were similar and consistent with in vitro clearance values derived in the analysis of 50 pharmaceutical compounds (McGinnity et al., 2004). The results of the plasma protein binding assay indicated that the majority of the ToxCast Phase I chemicals are highly bound to plasma proteins.

As described previously (Rotroff et al., 2010b), the results from the in vitro pharmacokinetic assays were combined with IVIVE to predict chemical concentration in the blood at steady state for specific human exposure scenarios. Using reverse dosimetry, we calculated the oral doses that would result in a steady-state blood concentration equivalent to the AC50 or LEC value in each of the ToxCast assays. Due to the large number of chemicals examined and the high-throughput nature of the assays, the IVIVE modeling was limited to the oral route and a set of simplifying assumptions were made. Each chemical was assumed to have 100% oral bioavailability, and excretion was limited to hepatic metabolism and glomerular filtration. These assumptions should generally be conservative from a human health standpoint because lower absorption or additional routes of excretion would result in a lower estimate of the oral equivalent dose required to achieve a specific plasma Css. One exception would occur if there were active renal resorption, which would result in a higher plasma Css at a given dose. Alternatively, high biliary clearance of parent or enterohepatic recirculation could also play a factor. Second, our analysis is predicated on the assumption that plasma concentrations equivalent to in vitro AC50 or LEC values would produce responses in vivo. The concentration of free chemical in an in vitro assay that elicits a response may differ from the assigned AC50 or LEC value due to factors such as protein-lipid composition of the media and binding of the chemical to plastics (Blaauboer, 2010). Third, the pharmacokinetics and bioactivity were only evaluated for the parent compound. No attempt was made to evaluate biological activities and dosimetry of metabolites. Finally, as in many other in vitro pharmacology and toxicology studies, the AC50 and LEC values were used as the basis for estimating the oral equivalent doses. Given the goal of using these assays in prioritization and risk assessment, other methods such as benchmark dose analysis (Crump, 1995) may be needed to estimate the minimum concentration required to observe a biological effect above that seen in controls.

To evaluate the accuracy of the IVIVE modeling and the potential impact of the model assumptions, the IVIVE model predictions were compared with Css values derived from published pharmacokinetic information for 13 chemicals. The IVIVE-predicted Css values for six chemicals (2,4-dichlorophenoxyacetic acid, bisphenol A, cacodylic acid, carbaryl, oxytetracycline dihydrate, and triclosan) were comparable to Css values based on the published models (i.e., within one order of magnitude). The IVIVE modeling assumptions should bias the results toward overprediction (i.e., the assumption of 100% absorption and 0% extrahepatic metabolism both act to increase Css). For example, the IVIVE-derived Css values for oxytetracycline dihydrate overpredicted the published model by 7.8-fold, which can be explained by its low oral bioavailability (<10%), documented both by our permeability assay data and literature findings (Bjorklund and Bylund, 1991; Nielsen and Gyrd-Hansen, 1996). Importantly, the incorporation of permeability and red blood cell partitioning data to assess the impact of bioavailability and blood partitioning on a subset of the chemicals provided only minimal improvement in the IVIVE predictivity of the Css (Table 1).

Of the seven remaining chemicals that were greater than an order of magnitude different, the IVIVE model overpredicted the Css values for five chemicals (fenitrothion, lindane, parathion, picloram, and thiabendazole) and underpredicted for two (PFOS and PFOA). An inherent uncertainty exists in the calculation to determine hepatic clearance in IVIVE that may partially explain the low concordance for these chemicals. Restrictive hepatic clearance was assumed in the pharmacokinetic modeling, where the on/off kinetics of protein binding are slow enough to become rate limiting. However, for many chemicals, hepatic clearance is nonrestrictive, i.e., all chemical, free and bound, is available for metabolic clearance (i.e., Fub = 0.99). To evaluate the impact of these assumptions, the Css values were calculated using both restrictive and nonrestrictive hepatic clearance. Although the assumption of nonrestrictive hepatic clearance improved the concordance within this subset of chemicals (8/13 vs. 6/13), its use in the broader analysis across all the chemicals would increase the number of underpredicted Css values. Triclosan is an example of a chemical that appears to have restrictive hepatic clearance. Picloram appears to have nonrestrictive hepatic clearance while the behavior of lindane appears as an intermediate between the two clearance assumptions (Table 1). For the two chemicals where the Css values were significantly underpredicted using both assumptions—PFOS and PFOA—the discrepancy is
likely due to active renal resorption which was not incorporated in the IVIVE model (Andersen et al., 2006; Loccisano et al., 2010).

To incorporate human variability in the IVIVE modeling, Monte Carlo simulations were performed based on the interindividual variability in a population of 100 healthy individuals of both sexes from 20 to 50 years of age (Rostami-Hodjegan and Tucker, 2007). The population-based $C_{50}$ values were then used to generate oral equivalent doses for each chemical across all the more than 500 ToxCast assays that possessed a measurable AC$_{50}$ or LEC value. The oral equivalent doses returned for the most sensitive fifth percentile

---

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Assay endpoint</th>
<th>AC$_{50}$ or LEC (µM)</th>
<th>Oral equivalent dose (mg/kg/day)$^a$</th>
<th>Human exposure (mg/kg/day)$^b$</th>
<th>AER (Or Eq/Hum exp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Phenylphenol</td>
<td>PTGER2 downregulation</td>
<td>4.4444</td>
<td>0.056900</td>
<td>0.2500</td>
<td>0.2276</td>
</tr>
<tr>
<td>2-Phenylphenol</td>
<td>Competitive binding of G protein–coupled receptor P2RY1</td>
<td>4.9400</td>
<td>0.063300</td>
<td>0.2500</td>
<td>0.2532</td>
</tr>
<tr>
<td>Acifluorfen</td>
<td>Change in SLC01B1 expression, 24 h</td>
<td>0.00636</td>
<td>0.000056</td>
<td>0.00013</td>
<td>0.4310</td>
</tr>
<tr>
<td>Chlorpropham</td>
<td>PLA2 downregulation</td>
<td>1.4815</td>
<td>0.002900</td>
<td>0.005</td>
<td>0.5800</td>
</tr>
<tr>
<td>Cyprodinil</td>
<td>Change in CYP1A2 expression, 48 h</td>
<td>10.474</td>
<td>0.020499</td>
<td>0.0257</td>
<td>0.7976</td>
</tr>
<tr>
<td>Cyprodinil</td>
<td>Change in CYP2B6 expression, 24 h</td>
<td>11.428</td>
<td>0.022366</td>
<td>0.0257</td>
<td>0.8703</td>
</tr>
<tr>
<td>Cyprodinil</td>
<td>Change in HMGCSS2 expression, 24 h</td>
<td>8.8588</td>
<td>0.017338</td>
<td>0.0257</td>
<td>0.67463</td>
</tr>
<tr>
<td>Cyprodinil</td>
<td>Change in SULT2A1 expression, 24 h</td>
<td>9.6951</td>
<td>0.018974</td>
<td>0.0257</td>
<td>0.7383</td>
</tr>
<tr>
<td>Cyprodinil</td>
<td>Change in UGT1A1 expression, 24 h</td>
<td>9.973</td>
<td>0.019518</td>
<td>0.0257</td>
<td>0.7595</td>
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<tr>
<td>Cyprodinil</td>
<td>Competitive binding of muscarinic receptor M2</td>
<td>12</td>
<td>0.023485</td>
<td>0.0257</td>
<td>0.9138</td>
</tr>
<tr>
<td>Cyprodinil</td>
<td>Competitive binding of muscarinic receptor M5</td>
<td>9.3</td>
<td>0.018201</td>
<td>0.0257</td>
<td>0.7082</td>
</tr>
<tr>
<td>Dicamba</td>
<td>Change in SLC01B1 expression, 24 h</td>
<td>0.0168</td>
<td>0.001200</td>
<td>0.0297</td>
<td>0.0404</td>
</tr>
<tr>
<td>Dichloran</td>
<td>CYP2A2 inhibition (rat)</td>
<td>0.058</td>
<td>0.00011</td>
<td>0.000375</td>
<td>0.2933</td>
</tr>
<tr>
<td>Fenbuconazole</td>
<td>Change in cell growth kinetics</td>
<td>0.0468</td>
<td>0.00156</td>
<td>0.000203</td>
<td>0.7685</td>
</tr>
<tr>
<td>Fenbuconazole</td>
<td>CYP2A2 inhibition (rat)</td>
<td>0.0139</td>
<td>0.00046</td>
<td>0.000203</td>
<td>0.2266</td>
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<tr>
<td>Fenbuconazole</td>
<td>CYP2B1 inhibition (rat)</td>
<td>0.0441</td>
<td>0.00147</td>
<td>0.000203</td>
<td>0.7241</td>
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<td>Fenhexamid</td>
<td>Change in CYP3A4 expression, 48 h</td>
<td>0.01654</td>
<td>0.016547</td>
<td>0.0452</td>
<td>0.3661</td>
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<tr>
<td>Fludioxonil</td>
<td>Change in cell growth kinetics</td>
<td>0.04677</td>
<td>0.01808</td>
<td>0.0264</td>
<td>0.6848</td>
</tr>
<tr>
<td>Fluroxypyr-meptyl</td>
<td>Increased CXCL9 secretion by dermal fibroblasts</td>
<td>1.4815</td>
<td>0.00498</td>
<td>0.0141</td>
<td>0.3532</td>
</tr>
<tr>
<td>Fluroxypyr-meptyl</td>
<td>Increased CSF1 secretion by dermal fibroblasts</td>
<td>1.4815</td>
<td>0.00498</td>
<td>0.0141</td>
<td>0.3532</td>
</tr>
<tr>
<td>Fluroxypyr-meptyl</td>
<td>Increased CCL2 secretion by keratinocyte/fibroblasts</td>
<td>1.4815</td>
<td>0.00498</td>
<td>0.0141</td>
<td>0.3532</td>
</tr>
<tr>
<td>Fluroxypyr-meptyl</td>
<td>Increased CXCL10 secretion by keratinocyte/fibroblasts</td>
<td>1.4815</td>
<td>0.00498</td>
<td>0.0141</td>
<td>0.3532</td>
</tr>
<tr>
<td>Fluroxypyr-meptyl</td>
<td>Increased MMP9 secretion by keratinocyte/fibroblasts</td>
<td>1.4815</td>
<td>0.00498</td>
<td>0.0141</td>
<td>0.3532</td>
</tr>
<tr>
<td>Fluroxypyr-meptyl</td>
<td>Inhibition of BACE1 activity</td>
<td>3.7100</td>
<td>0.01248</td>
<td>0.0141</td>
<td>0.8851</td>
</tr>
<tr>
<td>Isoxaben</td>
<td>Change in CYP3A4 expression, 24 h</td>
<td>0.22457</td>
<td>0.00612</td>
<td>0.0083</td>
<td>0.7373</td>
</tr>
<tr>
<td>Piperonyl butoxide</td>
<td>CYP2J2 inhibition</td>
<td>0.078</td>
<td>0.01607</td>
<td>0.0185</td>
<td>0.869</td>
</tr>
<tr>
<td>Prometon</td>
<td>Change in cell growth kinetics</td>
<td>0.40738</td>
<td>0.01119</td>
<td>0.016</td>
<td>0.6994</td>
</tr>
<tr>
<td>Pyraclostrobin</td>
<td>Mitochondrial membrane potential, 1 h</td>
<td>0.0391</td>
<td>0.00755</td>
<td>0.0083</td>
<td>0.9096</td>
</tr>
<tr>
<td>Quinlorac</td>
<td>Change in cell growth kinetics</td>
<td>0.0468</td>
<td>0.0006</td>
<td>0.0076</td>
<td>0.0789</td>
</tr>
<tr>
<td>Spirooxamine</td>
<td>Competitive binding of sigma receptor</td>
<td>0.025</td>
<td>0.00032</td>
<td>0.00241</td>
<td>0.1328</td>
</tr>
<tr>
<td>Tetracanazole</td>
<td>CYP2C19 inhibition</td>
<td>0.0088</td>
<td>0.00015</td>
<td>0.0008</td>
<td>0.1875</td>
</tr>
<tr>
<td>Triclosan</td>
<td>Change in CYP2B6 expression, 24 h</td>
<td>0.0347</td>
<td>0.01084</td>
<td>0.1350</td>
<td>0.0803</td>
</tr>
<tr>
<td>Triclosan</td>
<td>Change in cell growth kinetics</td>
<td>0.047</td>
<td>0.0146</td>
<td>0.1350</td>
<td>0.1082</td>
</tr>
<tr>
<td>Triclosan</td>
<td>Competitive binding of human norepinephrine transporter</td>
<td>0.314</td>
<td>0.1000</td>
<td>0.1350</td>
<td>0.7403</td>
</tr>
</tbody>
</table>

**Note.** Endpoints are based on assay findings using human targets unless noted otherwise. PLAU, urokinase-type plasminogen activator; PTGER2, prostaglandin E receptor.

$^a$Oral equivalent dose for the lower fifth percentile of a cohort of 100 healthy individuals of both sexes from 20 to 50 years of age.

$^b$Estimated aggregate human oral exposure from food and drinking water sources for the most highly exposed group or subpopulation.
of the population represent the amount this subgroup would need to consume on a daily basis to achieve a steady-state plasma concentration equivalent to the AC50 or LEC value. The rank order of the chemicals based on these oral equivalent doses (Figs. 3A–D) was significantly different from the order derived based on the AC50 or LEC values alone (Figs. 5A–D), indicating that incorporation of dosimetric information provides a necessary context in the interpretation of in vitro potency data generated in the HTS assays.

A comparison of oral equivalent values obtained from the ToxCast assay data with the upper limit of estimated human exposures identified 18 chemicals for which there were overlapping values. When compared against exposure estimates for the general U.S. population, the number of chemicals with overlapping oral equivalent doses was reduced to 10. Of the 18 chemicals that overlapped at the upper limit of human exposures, most were herbicides or fungicides (Table 3). The exposure estimates for many of these chemicals were based on the presence of residues on food crops, in livestock, or in drinking water. Two chemicals, 2-phenylphenol and triclosan, have both bactericidal and fungicidal properties and are found in common household items such as soap, toothpaste (triclosan), and disinfectant cleaners (2-phenylphenol). One chemical, piperonyl butoxide, is not a pesticide but serves as a synergist when combined with other pesticides due to its ability to inhibit cytochrome P450 enzymes and nonspecific esterases (Moores et al., 2009).

For nearly all the 18 chemicals for which there were overlapping oral equivalent doses and human exposure estimates, any known in vivo effects were extracted from the standard toxicological studies required for product registration. The registration studies primarily assess endpoints such as histopathology, clinical chemistry, and body weight. Few mechanistic studies have been performed on these chemicals. A comparison of the non-human in vivo effects with the most sensitive human in vitro assay endpoints is shown (Table 3). Among the in vivo effects for these chemicals, the most common was hepatotoxicity or liver tumors (10/18 chemicals). Among the in vitro assays, changes in cytochrome P450 activity or expression were observed in 44% (8/18) of the chemicals. It is important to note that chemically induced changes in the in vitro assays do not necessarily indicate a toxic or adverse response but rather indicate a biological perturbation potentially followed by adaptation and return to homeostasis or, alternatively, toxicity. Although some of the ToxCast in vitro assays have been associated with adverse in vivo responses (Judson et al., 2010; Kleinstreuer et al., 2011; Martin et al., 2011), all in vitro assays were weighted equally in this study. The definition of adverse responses based on in vitro assay results has been a source of significant debate in the toxicology community (Boekelheide and Andersen, 2010), and this debate is likely to continue with increased use of in vitro assays for prioritization and risk assessment.

AERs were determined for each chemical based on the oral equivalent dose at the lower bound of the range divided by either the estimated upper limit of human exposure or the exposure estimate for the general U.S. population (an AER < 1 would therefore indicate exposure sufficient to cause...
FIG. 5. Distribution of AC₅₀ and LEC values across the ~500 ToxCast assays for the ToxCast Phase I chemicals analyzed in this study. See Figure 3 for box-and-whisker format details. The chemicals are presented in the same order as Figures 3A–D to allow comparison of the two sets of figures.
FIG. 5. Continued.
bioactivity). Across all chemicals, the AERs derived for the general U.S. population were log-normally distributed with 50% of the chemicals having an AER > 123.03 and 75% having an AER > 11.48 (Fig. 4A). The median AER derived using estimates for the most highly exposed subpopulations was 44.67, with 75% of the AERs > 6.03 (Fig. 4B). Among the 18 chemicals identified in this study with overlapping oral equivalent doses and human exposure estimates, the AERs

**TABLE 3**


<table>
<thead>
<tr>
<th>Chemical</th>
<th>Use pattern</th>
<th><em>In vivo</em> effects</th>
<th>Assay endpoint hits</th>
<th>AER value/range</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Phenylphenol</td>
<td>Microbicide, bactericide (household), and fungicide (citrus, pears)</td>
<td>Bladder carcinogen (rat); liver carcinogen (mouse)</td>
<td>PTGER2 downregulation; competitive binding of G protein-coupled receptor P2RY1</td>
<td>0.23–0.25</td>
</tr>
<tr>
<td>Acifluorfen</td>
<td>Diphenyl ether herbicide (soybeans)</td>
<td>Liver carcinogen (mouse); kidney lesions</td>
<td>Change in SLCO1B1 expression, 24 h</td>
<td>0.4310</td>
</tr>
<tr>
<td>Chlorpropham</td>
<td>Herbicide (potatoes)</td>
<td>Thyroid toxicity</td>
<td>PLAU downregulation</td>
<td>0.5800</td>
</tr>
<tr>
<td>Cypnodinil</td>
<td>Fungicide (almonds, grapes, pome, and stone fruits)</td>
<td>Liver and kidney effects</td>
<td>Changes in expression of multiple cytochrome P-450s and UGT1A1; competitive binding of muscarinic receptors</td>
<td>0.67–0.91</td>
</tr>
<tr>
<td>Dicamba</td>
<td>Benzoic acid postemergent herbicide (broadleaf weeds, asparagus, corn, and oats)</td>
<td>Reproductive toxicity</td>
<td>Change in SLCO1B1 expression, 24 h</td>
<td>0.0404</td>
</tr>
<tr>
<td>Dichloran</td>
<td>Pre- and post-harvest fungicide (lettuce, peaches, and sweet potatoes)</td>
<td>Neuropathology; liver, kidney, spleen, and hematologic effects</td>
<td>CYP2A2 inhibition (rat)</td>
<td>0.1846</td>
</tr>
<tr>
<td>Fenbuconazole</td>
<td>Triazole fungicide (wheat, barley, apple, and pear)</td>
<td>Thyroid carcinogen; liver carcinogen</td>
<td>Change in cell growth kinetics; CYP2A2 inhibition (rat); and CYP2B1 inhibition (rat)</td>
<td>0.23–0.77</td>
</tr>
<tr>
<td>Fenhexamid</td>
<td>Fungicide (grapes, strawberries, and ornamentals)</td>
<td>Hematologic effects; adrenal changes</td>
<td>Change in CYP3A4 expression, 48 h</td>
<td>0.3660</td>
</tr>
<tr>
<td>Fludioxonil</td>
<td>Pyrolyle fungicide (citrus, apple, and root vegetables)</td>
<td>Decrease body weight; liver, kidney effects</td>
<td>Change in cell growth kinetics</td>
<td>0.6848</td>
</tr>
<tr>
<td>Fluoroxypyr-meptyl</td>
<td>Pyridine herbicide (barley, wheat, apple, and pear)</td>
<td>Decrease body weight; kidney effects</td>
<td>Increased CXCL9 secretion by dermal fibroblasts; increased CSF1 secretion by dermal fibroblasts; increased CXCL10 secretion by keratinocyte/fibroblasts; increased MMP9 secretion by keratinocyte/fibroblasts; and inhibition of human BACE1 activity</td>
<td>0.35–0.89</td>
</tr>
<tr>
<td>Isoxaben</td>
<td>Benzamizole herbicide (nonbearing fruit/nut trees, and Christmas trees)</td>
<td>Liver effects; enzyme induction</td>
<td>Change in CYP3A4 expression</td>
<td>0.7284</td>
</tr>
<tr>
<td>Piperonyl butoxide</td>
<td>Synergist; CYP450 and nonspecific esterase inhibitor</td>
<td>Hematologic effects and hepatotoxicity</td>
<td>CYP2J2 inhibition</td>
<td>0.869</td>
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<tr>
<td>Prometon</td>
<td>Herbicide (nonfood use); drinking water contaminant</td>
<td>Decrease maternal body weight gain</td>
<td>Change in cell growth kinetics</td>
<td>0.6994</td>
</tr>
<tr>
<td>Pyraclostrobin</td>
<td>Stroblurin fungicide (strawberries, onions, and corn)</td>
<td>Neurotoxicity; liver and kidney effects</td>
<td>Change in mitochondrial membrane potential</td>
<td>0.9096</td>
</tr>
<tr>
<td>Quinclorac</td>
<td>Quinoline carboxylic acid</td>
<td>Decrease in body weight</td>
<td>Change in cell growth kinetics</td>
<td>0.0789</td>
</tr>
<tr>
<td>Spiroxamine</td>
<td>Foliar fungicide (grapes cereals, and bananas)</td>
<td>Neurotoxicity, liver effects</td>
<td>Competitive binding of sigma receptor</td>
<td>0.1328</td>
</tr>
<tr>
<td>Tetracnazololate</td>
<td>Triazole fungicide</td>
<td>Mouse liver carcinogen</td>
<td>CYP2C19 inhibition</td>
<td>0.1875</td>
</tr>
<tr>
<td>Triclosan</td>
<td>Bactericide and fungicide (household items)</td>
<td>Thyroid effects</td>
<td>Change in CYP2B6 expression, 24 h; change in cell growth kinetics; competitive binding to norepinephrine transporter</td>
<td>0.08–0.74</td>
</tr>
</tbody>
</table>
ranged from 0.04 to 0.9 (Table 3). It is important to note that for some chemicals, the AER may be skewed due to the conservative nature of the human exposure estimate derivation in the registration documents. For chemicals with limited residue or exposure information, EPA uses a tiered approach with the first tier providing the most conservative exposure estimation. As actual exposure or residue levels are determined, the exposure estimates are refined.

The transition to a new toxicity testing paradigm that relies heavily on in vitro HTS assays will require a parallel investment in characterizing the pharmacokinetics and exposure levels of these chemicals. This parallel effort will add valuable information on parameters critical to interpreting biologically relevant exposure scenarios that should yield more informative prioritization models. The results and approach outlined in this study are part of that parallel effort and provide an important bridge between the nominal in vitro assay concentrations and the human oral equivalent doses required to achieve those concentrations in the blood. The subsequent comparison of the oral equivalent doses with human exposure estimates provides a better basis for informed decisions on chemical testing priorities and regulatory attention (Blaauboer, 2010; Cohen Hubal et al., 2010). Continued refinement of the in vitro assays to better reflect in vivo adverse effects and improvement in the suite of in vitro pharmacokinetic assays and IVIVE modeling will eventually allow us to move beyond hazard-based prioritization to risk assessment (Bhattacharya et al., 2011; Judson et al., 2011).

SUPPLEMENTARY DATA
Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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