Incorporating Human Dosimetry and Exposure into High-Throughput *In Vitro* Toxicity Screening

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Many chemicals in commerce today have undergone limited or no safety testing. To reduce the number of untested chemicals and prioritize limited testing resources, several governmental programs are using high-throughput *in vitro* screens for assessing chemical effects across multiple cellular pathways. In this study, metabolic clearance and plasma protein binding were experimentally measured for 35 ToxCast phase I chemicals. The experimental data were used to parameterize a population-based *in vitro*-to-*in vivo* extrapolation model for estimating the human oral equivalent dose necessary to produce a steady-state *in vivo* concentration equivalent to *in vitro* AC_{50} (concentration at 50% of maximum activity) and LEC (lowest effective concentration) values from the ToxCast data. For 23 of the 35 chemicals, the range of oral equivalent doses for up to 398 ToxCast assays was compared with chronic aggregate human oral exposure estimates in order to assess whether significant *in vitro* bioactivity occurred within the range of maximum expected human oral exposure. Only 2 of the 35 chemicals, triclosan and pyrithiobac-sodium, had overlapping oral equivalent doses and estimated human oral exposures. Ranking by the potencies of the AC_{50} and LEC values, these two chemicals would not have been at the top of a prioritization list. Integrating both dosimetry and human exposure information with the high-throughput toxicity screening efforts provides a better basis for making informed decisions on chemical testing priorities and regulatory attention. Importantly, these tools are necessary to move beyond hazard rankings to estimates of possible *in vivo* responses based on *in vitro* screens.

Key Words: reverse dosimetry; human exposure; ToxCast; high-throughput screening.

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The current paradigm for testing agricultural and industrial chemicals for potential human health effects is inefficient, expensive, and relies heavily on experimental animals (Andersen and Krewski, 2009; Holsapple *et al.*, 2009; NRC, 2007). Tests typically include evaluation of carcinogenicity and chronic, developmental, and reproductive toxicities (Chhabra *et al.*, 2003; Martin *et al.*, 2009). Because of the complexity and expense of these tests and the current regulatory requirements that dictate product registration, most chemicals currently in commerce have undergone only limited or no safety testing (Allanou *et al.*, 2003; EPA, 1998; Judson *et al.*, 2009; Locke and Myers, 2010; Wilson and Schwarzman, 2009). This deficit in safety testing is beginning to be addressed through legislative mandates. In Europe, the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) initiative began a phased implementation in 2007 that will substantially alter the safety testing performed on both new and existing chemicals (Foth and Hayes, 2008; Wilson and Schwarzman, 2009). In the United States, legislation to overhaul the Toxic Substances Control Act is currently under discussion in Congress (Locke and Myers, 2010). Nonetheless, legislative action requiring safety testing on more chemicals using the traditional testing paradigm will significantly increase the economic burden and animal use while not addressing many of the issues that exist within the current system. The regulations under REACH have been estimated to directly cost industry $4.2 billion (Brown, 2003) and require the use of more than 45 million animals over the next 15 years (Breithaupt, 2006; Hofer *et al.*, 2004).

To address the large number of untested chemicals and improve chemical risk management, the U.S. Environmental Protection Agency (EPA) developed a high-throughput testing program called ToxCast to screen chemicals and prioritize limited testing resources toward those representing the greatest potential risk to human health (Dix *et al.*, 2007). In the first
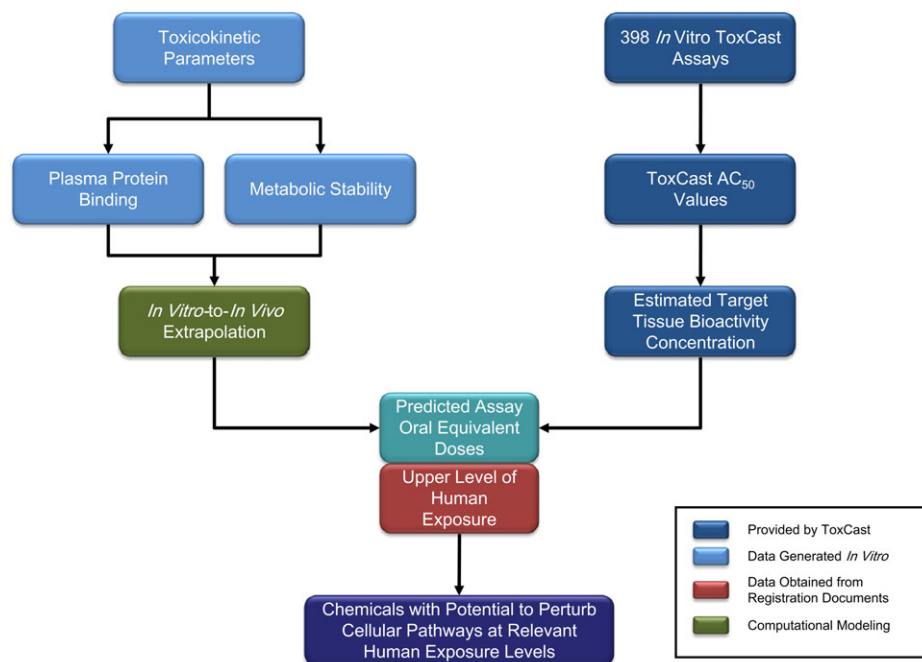
FIG. 1. Schematic representation for incorporating human dosimetry and exposure information into the high-throughput *in vitro* toxicity screening process performed as a part of ToxCast.

phase of the ToxCast program, hundreds of *in vitro* assays were used to screen a library of agricultural and industrial chemicals to identify cellular pathways and processes perturbed by these chemicals (Judson *et al.*, 2010). However, the use of *in vitro* assay potencies alone for prioritizing chemicals for testing may over- or underestimate the potential risk of these chemicals because of differences in bioavailability, clearance, and exposure *in vivo* (Blaauboer, 2010).

To evaluate the utility of incorporating human dosimetry and exposure into high-throughput *in vitro* toxicity screening, a combination of experimental assays, computational modeling, and exposure assessment were performed on a subset of 35 ToxCast chemicals (Fig. 1). For human dosimetry, *in vitro* assays were used to estimate metabolic clearance and plasma protein binding. Computational *in vitro*-to-*in vivo* extrapolation methods were then used to estimate the human oral equivalent doses that would be required to produce steady-state *in vivo* concentrations equivalent to *in vitro* AC_{50} (concentration at 50% of maximum activity) and LEC (lowest effective concentration with statistically significant increase in activity above the negative controls) values from the ToxCast data. These oral equivalents were compared with human oral exposure estimates based on maximally allowed chemical residue levels from food and drinking water. By comparing the human oral equivalent doses across all the ToxCast assays with the exposure estimates, two chemicals with the potential to perturb cellular pathways at estimated human oral exposure levels were identified, suggesting that further review of these chemicals may be warranted.

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 35 chemicals in this study are a subset of the ToxCast phase I chemical library (http://www.epa.gov/ncct /toxcast/chemicals.html). The subset was selected based on pilot studies measuring cellular ATP levels as an indicator of cytotoxicity. ToxCast phase I studied a chemical library of 320 substances (Judson *et al.*, 2010). Within that set, there are 309 unique chemicals, most of which are food-use pesticides for which extensive animal testing results are available. The mechanisms of toxicity for a number of these chemicals are known, thus affording the opportunity to match *in vitro* results with existing knowledge. Chemical information was quality reviewed and structure annotated within the DSSTox database project (for more information on DSSTox quality review procedures and standard chemical fields, see: http://www.epa.gov/ncct/dsstox).

The 35 chemicals were obtained from various vendors with all but three possessing a purity exceeding 97% (the samples of emamectin benzoate, MGK-264, and propetamphos were 95, 92.7, and 96.8% pure, respectively). The specific vendor and purity information for each chemical is provided as Supplementary table 1. Stock solutions (20mM) were prepared in DMSO and stored in amber vials at −80°C. The stock solutions were diluted to the 0.2 and 2mM working concentrations at the time the assays were conducted.

Plasma protein binding assay. Plasma protein binding was estimated for each of the 35 chemicals using the rapid equilibrium dialysis (RED) method (Waters *et al.*, 2008). Human plasma was recovered from whole blood donations (using anticoagulant K_2EDTA) obtained from healthy, consented paid human donors at a U.S. Food and Drug Administration-licensed and inspected donor center (catalog number HMPLEDTA2; Bioreclamation, Inc.,

Westbury, NY). The plasma was pooled from three male (29, 49, and 53 years old) and three female (23, 31, and 38 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 $2000 \times g$ for 10 min prior to analysis. RED was conducted in 96-well format using the single-use RED plate (catalog number 90006; Pierce Biotechnology, Rockford, IL) according to manufacturer's instructions. PBS and plasma were loaded to the buffer and sample chambers, respectively, using a Biomek FX workstation (Beckman Coulter, Brea, CA). Chemical stock solutions were added to the sample chambers to achieve final concentrations of 1 and 10mM. 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 in an orbital shaker for 4 h. Aliquots were removed from the buffer and sample chambers, mixed with acetonitrile (100%) to precipitate proteins, and stored at −80°C until analytical analysis. The binding assays were performed in triplicate.

Metabolic clearance assay. The rate of hepatic metabolism of the parent chemical was measured at two concentrations (1 and 10µM) over a 2-h period using cryopreserved primary human hepatocytes (CellzDirect/Invitrogen Corporation, a part of Life Technologies, Durham, NC). The 1µM concentration was chosen because it is a standard concentration used in metabolic stability studies 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, and they provide sufficient concentrations of chemical at the later time points to ensure detection in the analytical analysis. The hepatocytes were obtained from two separate pools of 10 individual donors (HuP50 and HuP2000). 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 and viability, and all values fell within acceptable ranges based on standard CellzDirect/Invitrogen Corporation quality control standards. 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.

Incubation medium was prepared by supplementing William's E Medium with 0.1µM dexamethasone, 1 ml ITS+ (per 500 ml medium), 4mM Glutamax, 15mM HEPES, and either 2 µg/ml gentamycin or 2.5 ml penicillin/streptomycin (per 500 ml medium). In separate amber glass vials, the chemical stocks were added to warm incubation medium to yield the targeted working concentrations (e.g., 2µM by adding 10 µl 1mM chemical stock to 5 ml of incubation medium). The incubation media containing the chemical (0.5 ml) was added to a single well of a 12-well noncoated polystyrene plate. The plates were then placed in an incubator at 37°C/5% CO_2 on an orbital shaker prior to addition of the cells.

Vials of cryopreserved hepatocytes were thawed using warm William's E Medium (37°C) and centrifuged at $76 \times g$ for 6 min at 20°C. The cells were resuspended in incubation medium to a working density of 1.0×10^6 viable cells per milliliter as determined by Trypan Blue exclusion. To each well of the incubation plates, 0.5 ml of 1.0×10^6 viable cells per milliliter were added to yield a final cell density of 0.5×10^6 viable cells per milliliter. An aliquot of the hepatocytes was boiled for 5 min (heat treated) to denature proteins prior to addition to the incubation medium. The boiled hepatocytes served as a negative matrix control for each chemical. A media-only negative control was also included for each chemical. The plates were maintained in the incubator on an orbital shaker at a set speed of 120 rpm for the duration of the incubations. At time points of 0, 15, 30, 60, and 120 min, 50 µl aliquots were removed from each well and quenched with 50 µl ice cold acetonitrile. Duplicate wells were run for each chemical. The quenched aliquots were frozen at −70°C prior to analysis.

Analytical chemistry analysis by LC/MS. Samples from the metabolic clearance assay were thawed at room temperature, vortexed briefly, and centrifuged at $6000 \times g$ for 1 min. Samples were then diluted 1:10 with 5% vol/vol acetonitrile in water, mixed, and centrifuged for 1 min at $6000 \times g$. Samples were analyzed on an API 3000 Liquid Chromatography/Mass Spectrometry (LC/MS) (Applied Biosystems, Foster City, CA). Specific instrument parameters for each analyte are provided as Supplementary table 2.

Samples from the plasma protein binding assay were thawed at room temperature, vortexed briefly, and centrifuged at $10,000 \times g$ for 10 min. Samples were diluted in 5% vol/vol acetonitrile in water, mixed, and centrifuged at $6000 \times g$ for 1 min prior to analysis on the API 3000 LC/MS. Specific instrument parameters for each analyte are provided as Supplementary table 2.

Analytical chemistry analysis by gas chromatography-mass spectrometry. All samples for gas chromatography-mass spectrometry analysis were thawed at room temperature, vortexed briefly, and centrifuged at $10,000 \times g$ for 10 min. Solid phase extraction (SPE) was conducted using Sep-Pak C_{18} 96-well SPE plates (catalog number 186003966; Waters, Milford, MA) in conjunction with a vacuum manifold following manufacturer's instructions. Samples were eluted in methanol into gas chromatography (GC) vials prior to loading on an Agilent 6890 GC with model 5973 MS (Agilent Technologies, Santa Clara, CA). Samples were analyzed using electron impact mode and selective ion monitoring. Specific instrument parameters for each analyte are provided as Supplementary table 3.

Plasma protein binding data analysis. To calculate percent of unbound chemical (F_{ub}), the test compound concentration in the chamber without plasma protein was divided by the concentration in the chamber containing plasma and multiplied by 100. If the concentration of the chemical in the free fraction was below the limit of detection, a default F_{ub} of 0.005 was assumed. The default value was estimated based on two standard deviations over the minimum amount of binding detected in a previous study (Waters *et al.*, 2008).

Metabolic clearance data analysis. Metabolic clearance for the 1 and 10µM starting concentrations were plotted separately in semilog format (log concentration vs. time) with two replicates at each time point. The concentration data at each time point for each chemical are provided as Supplementary table 4. The time-course data at each concentration were analyzed using linear regression. Based on the slope of the regression line, a clearance value was estimated and normalized to cell number. The units of clearance were microliter per minute per 10^6 cells. Considering two replicates at each of the five time points, a standard F-test ($df = 1,8$; $\alpha = 0.10$) 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 2-h time point, the degrees of freedom were adjusted accordingly. For chemicals that fell below detection levels before the 2-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. Chemicals that had no statistically significant change ($p > 0.10$) were assigned a metabolic clearance of 0 at the concentration examined.

In vitro bioactivity data. The ToxCast program measured activity of 309 compounds against a set of ~400 *in vitro* assays using nine separate technologies, including cell-based and cell-free binding assays, protein and RNA expression, cell imaging, and real-time electronic impedance measurements. Each chemical-assay combination was run in concentration response format and AC_{50} or LEC values were estimated. The *in vitro* bioactivity was assumed to be solely the result of the parent compound. Many assays were metabolically inactive, although the CellzDirect, Attagene, and Cellumen assays possessed some metabolic capacity. A detailed description of the assays and associated data are provided in an earlier publication (Judson *et al.*, 2010). All data are available from the ToxCast Web site (http://www.epa.gov/ncct/toxcast).

Estimation of oral equivalents using **in vitro-to-in vivo** *extrapolation.* Simulations were performed for each chemical and each AC_{50} or LEC value across 398 *in vitro* assays. The chemical and assay combinations that did not show activity (i.e., did not possess an AC_{50} or LEC value) were not simulated. For each compound, an empirical one-compartment pharmacokinetic (PK) model was parameterized and simulations were performed using the Simcyp software to predict *in vivo* kinetics based on *in vitro* data (Simcyp Limited, Sheffield, UK). In the model, there were two routes of elimination: metabolism and renal

excretion. Gastrointestinal absorption was assumed to be 100%. The model was assumed to describe the blood concentration, and therefore, the concentration predicted by the model is equivalent to the concentration of compound in the blood flowing through the kidney. Unbound parent compound was assumed to be available for secretion by glomerular filtration into the renal tubules where it is not reabsorbed and instead passes into urine (Rowland *et al.*, 1973). The rate of renal excretion was estimated by multiplying the measured F_{ub} by the normal adult human glomerular filtration rate (111 ml/min/1.73 m^2 = 6.7 l/h) (Rule *et al.*, 2004). Similarly, the concentration in blood was assumed to correspond to the concentration in liver and thus determine the amount of compound available for metabolism. The estimated rate of metabolism from *in vitro* kinetic studies with hepatocytes and the measured F_{ub} were input into the software application, which then converted it into the equivalent *in vivo* hepatic clearance using the estimated number of hepatocytes per gram liver and the liver weight. The extrinsic metabolic clearance was then calculated from the liver clearance and the liver blood flow. The two clearances (i.e., hepatic and renal) were used in the model to estimate the total clearance for the one-compartment model. Because the measured *in vitro* bioactivity was assumed to be because of parent compound alone, both metabolism and elimination have the same effect—they reduce the amount of parent compound.

For the metabolic clearance values, either the 1 or the 10μM value was used depending on which value was closer to the AC$_{50}$ or LEC concentration. The *in vitro* hepatocytes were assumed to maintain physiological rates of xenobiotic metabolism. For the plasma protein binding values, the F_{ub} was averaged across the 1 and 10μM concentrations and used as input. If the experimentally measured F_{ub} was below the limit of detection, then a default value of 0.005 was assumed. Because greater binding reduces clearance and increases steady-state concentrations, this assumption is conservative by reducing the oral equivalent dose necessary to achieve a given steady-state concentration.

The *in vitro* AC$_{50}$ or LEC values were assumed to correspond to blood concentrations sufficient for bioactivity *in vivo*. Tissue concentrations may be higher or lower than blood concentrations but require measurements of chemical- and tissue-specific partition coefficients. Some methods are available for predicting tissue partitioning based on compound structure or octanol-water partitioning (Payne and Kenny, 2002; Poulin and Theil, 2002), but these methods were not employed because of the breadth of chemicals analyzed in the study and the uncertainty associated with the predictions. It should be noted that for the one-compartment model, the steady-state concentration is independent of the volume of distribution.

Simulations were performed using a dose of 1 mg/kg/day. 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 (C_{ss}) were obtained as output. These values were then used as conversion factors to generate oral equivalent doses according to the following formula:

$$\text{Oral Equivalent (mg/kg/day)} = \text{ToxCast AC}_{50} \text{ or LEC (μM)} \times \frac{1 \text{ mg/kg/day}}{C_{ss} \text{(μM)}}.$$

Note that the oral equivalent value is linearly related to the *in vitro* AC$_{50}$ or LEC and the C_{ss}. If nonlinearities, e.g., Michaelis-Menten kinetics, were introduced into the model, a more complicated approach would be necessary. The generic model parameters are provided as Supplementary table 5.

Evaluation of PK modeling. Published PK data for triclosan (Gentry *et al.*, 2002; Kohli *et al.*, 1974; Sauerhoff *et al.*, 1977; Volkel *et al.*, 2002), 2,4-dichlorophenoxyacetic acid (Gentry *et al.*, 2002; Kohli *et al.*, 1974; Sauerhoff *et al.*, 1977; Volkel *et al.*, 2002), oxytetracycline dihydrate, and bisphenol A (Gentry *et al.*, 2002; Kohli *et al.*, 1974; Sauerhoff *et al.*, 1977; Volkel *et al.*, 2002), and a physiologically based pharmacokinetic (PBPK) model for parathion (Gentry *et al.*, 2002; Kohli *et al.*, 1974; Sauerhoff *et al.*, 1977;

Volkel *et al.*, 2002), were used to estimate the C_{ss} in the plasma in humans exposed to 1 mg/kg/day.

Estimation of human oral exposure. Except for bisphenol A, exposure estimates were obtained from available EPA Office of Pesticide Programs documents. 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. As a conservative estimate, the most highly exposed group or subpopulation was used. The exposure estimates for each chemical are provided as Supplementary table 6. Estimated exposure for bisphenol A was calculated using the urinary concentration of total bisphenol A, as determined in the National Health and Nutrition Examination Survey (NHANES). Concentrations were then back calculated by multiplying by 24-h urinary output volume (NTP, 2008). The highest exposed subgroup, ages 12–19 years, was used in the data analysis.

RESULTS

Plasma Protein Binding

Measurements of plasma protein binding were performed at 1 and 10μM for each chemical. Because of the minimal differences observed between the F_{ub} values at 1 and 10μM and that binding to plasma proteins is unlikely to be saturated at the concentrations tested, the values were averaged to obtain a single F_{ub} for each chemical. The results from the plasma protein binding measurements showed that most chemicals were highly bound to plasma protein (Fig. 2A). For four chemicals (etoxazole, MGK, parathion, and triclosan), no unbound chemical was detected at either the 1 or the 10μM concentrations. It is possible that these chemicals were bound to the membrane present in the dialysis device thereby reducing the amount of unbound chemical.

Metabolic Clearance

The metabolic clearance measurements ranged from 250.3 to −12.3 μl/min/10^6 cells. The negative values may reflect lack of or little-to-no metabolism, a very slow rate of metabolism, or variability in the experimental measurements. A total of 19 chemicals showed either no metabolism or saturation at the 10μM concentration. Of these chemicals, only eight (2,4-dichlorophenoxyacetic acid, acetamiprid, bentazone, bromacil, emamectin benzoate, isoxaben, oxytetracycline dihydrate, and pyrithiobac-sodium) were also saturated at the 1μM concentration. The distribution of metabolic clearance measurements was similar to the plasma protein binding measurements (Fig. 2B); however, no significant correlation was present between metabolic clearance and F_{ub} ($r^2 = 0.007$).

In Vitro-*to*-In Vivo *Extrapolation Modeling*

To evaluate the performance of the *in vitro*-to-*in vivo* modeling, the results for five ToxCast chemicals were compared with published PK data and PBPK model predictions (Table 1). For triclosan, 2,4-dichlorophenoxyacetic acid, and parathion, the
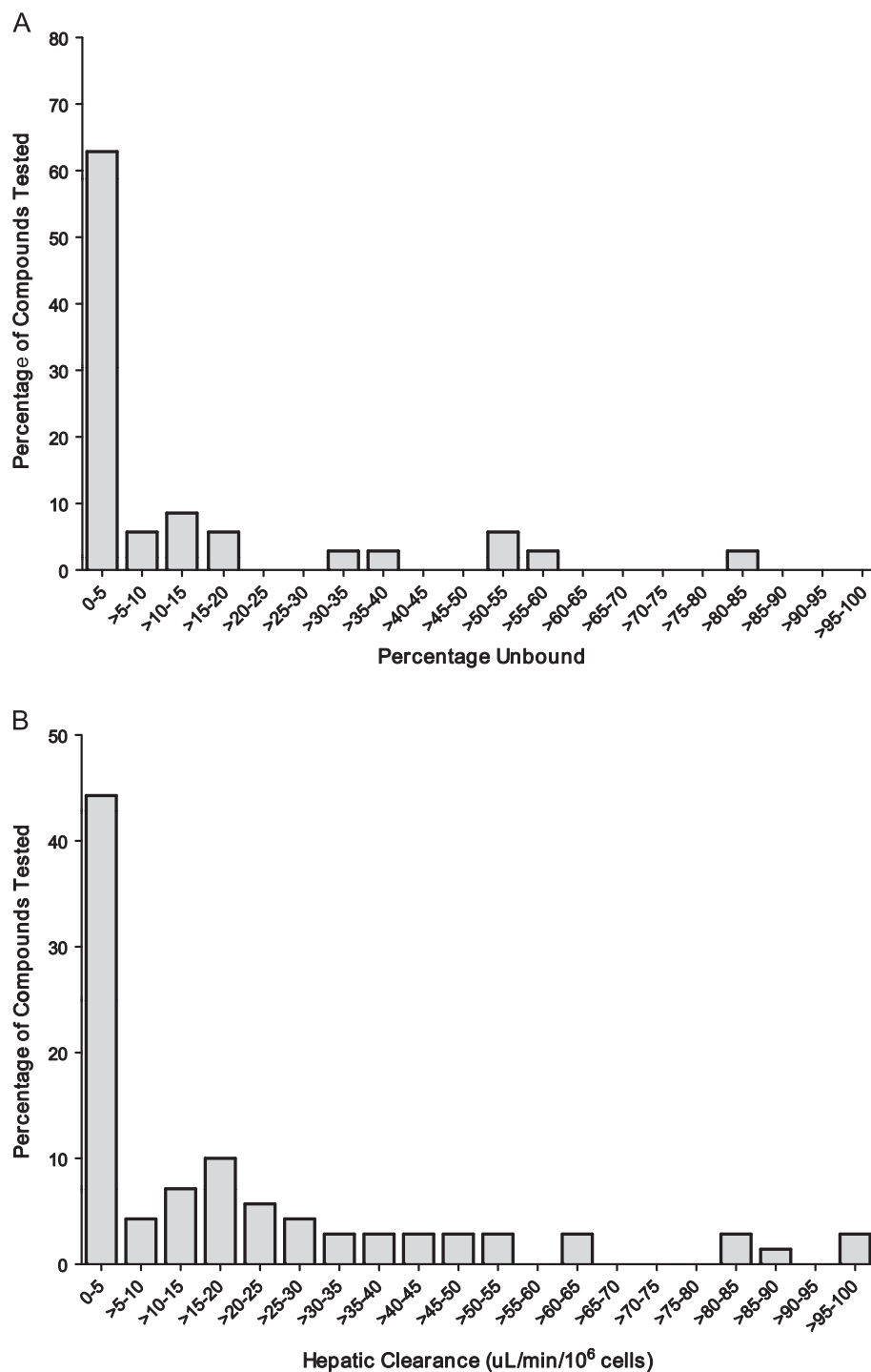
A

B

FIG. 2.   Distribution of (A) plasma protein binding measurements (averaged for both 1 and 10μM) and (B) metabolic clearance measurements (includes both the 1 and the 10μM concentrations) for the 35 ToxCast chemicals analyzed.

in vitro predicted C_{ss} value were similar to the human kinetic studies and published PBPK model. However, the PBPK model used to compare the results for parathion (Gentry *et al.*, 2002) did not include binding of parathion in the blood or excretion of parathion in the urine. Therefore, to make a consistent comparison between the PBPK and the *in vitro*-to-*in vivo* modeling, the F_{ub} was set to 0.99 with no renal clearance. For oxytetracycline dihydrate, the *in vitro* model overpredicted the C_{ss} value calculated from human PK data. One explanation for this overprediction is the assumption of 100% oral absorption in

TABLE 1
Comparison of *In Vitro*-to-*In Vivo* Extrapolation Modeling Results with Published PK or PBPK Models

Chemical	PK- or PBPK -derived $C_{ss}{}^{a}$ (μM)	*In vitro*-to-*in vivo* extrapolation $C_{ss}{}^{a,b}$ (μM)
2,4-Dichlorophenoxyacetic acid	9.05–90.05	40.77
Oxytetracycline dihydrate	0.36	2.80[c]
Triclosan	2–10	1.49
Bisphenol A	< 0.13	0.42
Parathion	0.17	0.14[d]

[a]C_{ss}, concentration at steady state for 1 mg/kg/day dose.
[b]Predicted using the 1μM metabolic clearance rate.
[c]Does not consider low oral bioavailability.
[d]Modeled with $F_{ub} = 0.99$ and $Cl_{renal} = 0$ to match PBPK validation model (Gentry *et al.*, 2002).

the model. By comparison, the oral bioavailability of oxytetracycline dihydrate has been reported to be less than 10% (Bjorklund and Bylund, 1991; Nielsen and Gyrd-Hansen, 1996). The comparison for bisphenol A is complicated by the fact that the *in vitro* prediction represents an estimate of the free concentration of the parent compound in the plasma, whereas the experimental data were for total bisphenol A, including the glucuronide (Volkel *et al.*, 2002). The authors estimated that bisphenol A in the plasma was greater than 99% glucuronidated. To allow an accurate comparison, the C_{ss} for total bisphenol A calculated from the published study was divided by 100 to estimate the free concentration.

Using the *in vitro*-to-*in vivo* modeling approach, oral equivalent doses were determined for each of the 35 chemicals across all of the 389 ToxCast assays that possessed a measurable AC_{50} or LEC value. For example, among the 35 chemicals, emamectin benzoate had AC_{50} or LEC values for 127 assays, whereas acetamiprid had AC_{50} or LEC values for six assays. In the modeling simulations, Monte Carlo analysis was performed to simulate variability in the C_{ss} across a cohort of 100 healthy individuals of both sexes from 20 to 50 years of age. From the Monte Carlo analysis, the upper 95th percentile of the C_{ss} was used to obtain a conservative estimate of the oral equivalent doses for a population. The lowest fifth percentile of the oral equivalent doses were summarized as box plots representing the range of values over all the ToxCast assays (Fig. 3). From the chemicals tested, emamectin benzoate had the lowest oral equivalent doses at the fifth percentile with a median of 10.8 μg/kg/day and a minimum of 0.61 μg/kg/day. The chemical that generated the highest oral equivalent doses at the fifth percentile was diazoxon with a median oral equivalent of 512.8 mg/kg/day. The complete results from the *in vitro*-to-*in vivo* computational modeling as well as the plasma protein binding values, metabolic stability values, and the assay AC_{50} or LEC value for each chemical are provided as Supplementary table 7.

Estimated Human Oral Exposures

Aggregate human oral exposure estimates based on maximally allowed chemical residue levels from food and drinking were obtained from registration documents and NHANES data for 23 of the 35 chemicals. For most chemicals, the estimated aggregate oral exposures were broken down by various age groups and subpopulations. As a conservative approach, the estimate for the most highly exposed group or subpopulation were used. When these exposure estimates were compared with the oral equivalent doses from the ToxCast assays, only two chemicals, pyrithiobac-sodium and triclosan, showed overlap in the values (Fig. 3). The exposure estimates for a third chemical, buprofezin, were close but did not overlap the oral equivalent doses. Table 2 lists the ToxCast assays that were activated by triclosan and pyrithiobac-sodium at levels below the highest estimated exposures. The highest estimated oral exposure for triclosan is 0.13 mg/kg/day. Out of a total of 86 ToxCast assays that produced AC_{50} or LEC values for triclosan, the CLZD_CYP2B6_24, ACEA_LOCdec, and NVS _TR_hNET assays overlapped with the estimated exposure because of oral equivalents of 0.0117, 0.0158, and 0.106 mg/kg/day, respectively. Therefore, oral exposures for the most highly exposed individuals were estimated to be occurring at 1.2- to 11.1-fold higher than predicted bioactivity. The highest estimated oral exposure for pyrithiobac-sodium is 0.0012 mg/kg/day. A total of 15 ToxCast assays produced AC_{50} or LEC values for pyrithiobac-sodium, with overlap for the CLZD_SLCO1B1_48 assay because of an oral equivalent of 0.00116 mg/kg/day. Therefore, the oral exposure for the most highly exposed individuals was approximately equal with the oral equivalent for that assay.

DISCUSSION

Over the past 5 years, there has been a far-reaching discussion on the future direction of toxicology and how chemical testing is performed (Collins *et al.*, 2008; Dix *et al.*, 2007; NRC, 2007). One strategy has focused on the use of high-throughput *in vitro* screening to assess responses across multiple assays and prioritize compounds for conventional *in vivo* testing (Dix *et al.*, 2007). Current efforts are underway in both the United States and the Europe to assess the utility of high-throughput *in vitro* methods to efficiently screen chemicals and prioritize limited testing resources (Abbott, 2009; Kavlock *et al.*, 2009; Knight, 2008). In these high-throughput screening activities, most of the initial effort has focused on characterizing the biological activity of agricultural and industrial chemicals across multiple cellular pathways and processes (Judson *et al.*, 2010). Less attention has been paid to determining the relationship between concentrations of the chemical active *in vitro* and expected concentrations in human populations (Verwei *et al.*, 2006). PK properties and human exposure characteristics are equally important as the biological activity in determining a chemical's risk to human
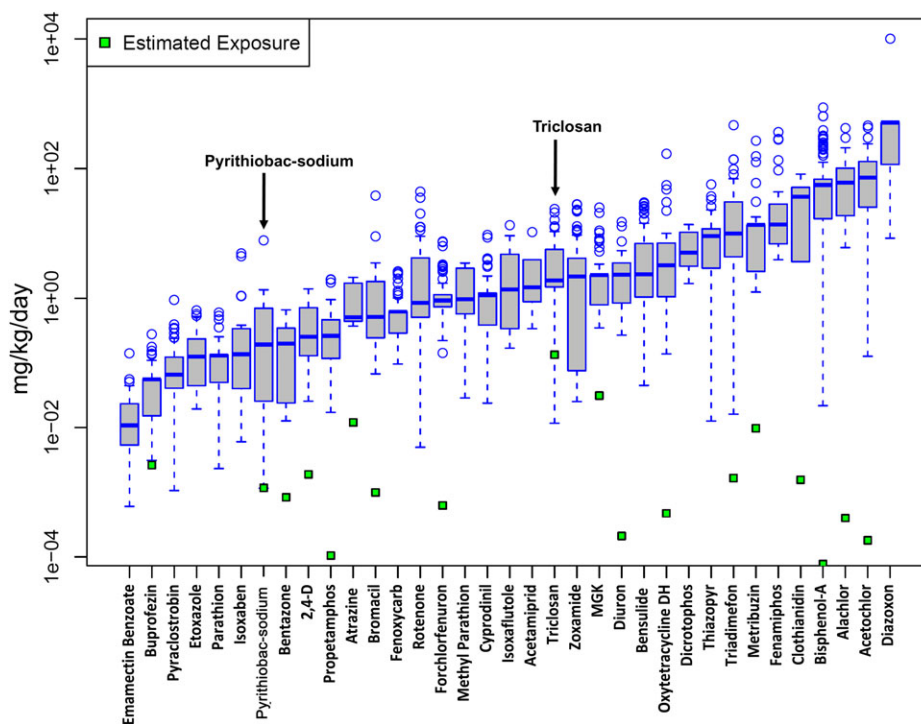
FIG. 3. Comparison of oral equivalent doses and highest estimated human oral exposure levels for the 35 ToxCast chemicals analyzed. The oral equivalent doses (milligram per kilogram per day) for each chemical were estimated for each of the 398 ToxCast assays that possessed a measurable AC_{50} value using *in vitro*-to-*in vivo* extrapolation modeling. In the modeling analysis, Monte Carlo simulation was performed to simulate variability across a cohort of 100 healthy individuals of both sexes from 20 to 50 years of age. From the Monte Carlo simulations, the lower fifth percentile of oral equivalent doses was selected as a conservative estimate for a population. The distribution of the oral equivalent doses is depicted as a box plot showing the median, upper, and lower 95% confidence intervals, with circles indicating data points outside the 95% range. Human exposure estimates for 23 chemicals were obtained from reregistration eligibility documents and NHANES biomonitoring data and reflect the most highly exposed group or subpopulation (green squares). Exposures for dicrotophos, fenamiphos, and methyl parathion were below 1×10^{-5} mg/kg/day and are therefore not shown on the graph. Chemicals where the highest estimated human oral exposure values fall within the range of predicted oral equivalents are highlighted with arrows.

health (Blaauboer, 2003; Cohen Hubal *et al.*, 2010; Krewski *et al.*, 2009).

In this study, *in vitro* assays were performed on a subset of ToxCast chemicals to estimate two critical determinants of PKs: metabolic clearance and plasma protein binding. The metabolic clearance of the chemicals was measured in cryopreserved

human hepatocytes, which retain the metabolic function of both cytochrome P450 (CYP450) and non-CYP450 enzymes (Li *et al.*, 1999). The use of cryopreserved human hepatocytes more accurately predicts the *in vivo* metabolic activity when compared with other methods, such as microsomal incubation (Jones and Houston, 2004). For the chemicals evaluated in this study, 27 of

TABLE 2

Chemicals and Corresponding *In Vitro* Assays with Oral Equivalent Doses Lower Than the Estimated Human Oral Exposure

Chemical	Assay	Endpoint	AC_{50} (μM)	Oral equivalent dose (mg/kg/day)[a]	Human exposure (mg/kg/day)[b]
Triclosan	CLZD_CYP2B6_24	CYP2B6 messenger RNA (mRNA) in primary human hepatocytes (24 h)	0.034	0.0117	0.13
Triclosan	ACEA_LOCdec	Cellular impedance measuring alterations in cell morphology and cell survival	0.046	0.0158	0.13
Triclosan	NVS_TR_hNET	Competitive binding of the human norepinephrine transporter	0.31	0.1057	0.13
Pyrithiobac-sodium	CLZD_SLC01B1_48	SLCO1B1 mRNA in primary human hepatocytes (48 h)	0.067	0.0012	0.0012

[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.
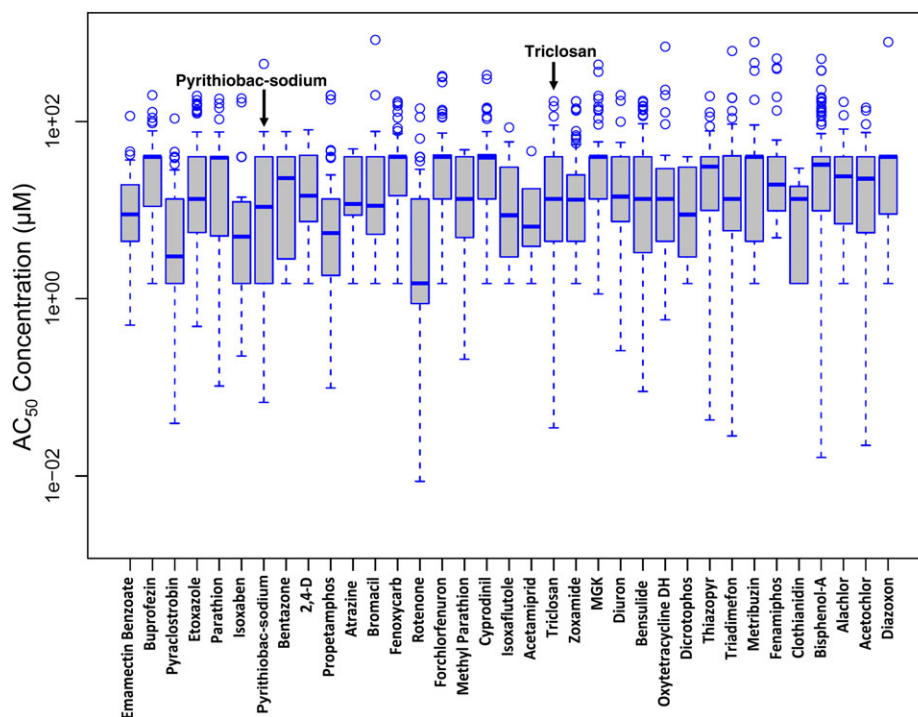
FIG. 4. Box plots of AC_{50} values across the 398 ToxCast assays for the 35 ToxCast chemicals analyzed. The median, upper, and lower 95% confidence intervals with circles indicating points outside the 95% span are indicated as in Figure 3. The chemicals are presented in the same order as Figure 3 for comparison purposes.

35 showed significant metabolic clearance in at least one of the two concentrations tested. Plasma protein binding was examined using RED, which has become a standard method for measuring binding in high-throughput studies (Waters *et al.*, 2008). Sixty-three percent of the 35 chemicals were more than 95% bound to plasma proteins. Based on this sample of chemicals, the results suggest that although most ToxCast chemicals are metabolically labile, binding to plasma proteins may limit availability for metabolism or renal clearance and would increase the effective half-life.

The metabolic clearance and plasma protein binding measurements were used together with *in vitro*-to-*in vivo* extrapolation modeling to predict the PK behavior of the chemicals at steady state (Shiran *et al.*, 2006). The use of *in vitro*-to-*in vivo* extrapolation modeling has been widely employed within the pharmaceutical industry to assess the preclinical PKs of candidate molecules (Caldwell *et al.*, 2009; De Buck and Mackie, 2007). In addition, Monte Carlo methods have been used together with *in vitro*-to-*in vivo* extrapolation modeling to simulate human variability within clinical trials (Rostami-Hodjegan and Tucker, 2007). Using *in vitro*-to-*in vivo* extrapolation modeling together with Monte Carlo simulation, population-based oral equivalent doses were determined for each of the 35 chemicals across all the 389 ToxCast assays that possessed a measurable AC_{50} or LEC value. For the purposes of this study, AC_{50} and LEC values were used as a measure of significant biological activity. Other values (e.g., AC_{20} or AC_{75}) could also be used. The range of oral equivalent doses for each

chemical represents the amount a person in the lowest fifth percentile would have to consume on a daily basis to achieve a steady-state plasma concentration equivalent to the AC_{50} or LEC value. The rank order of the chemicals based on the oral equivalent doses was significantly different from the order based on the AC_{50} and LEC values (Fig. 4), which suggests that prioritization based solely on AC_{50} and LEC values may misrepresent the potential hazard of chemicals.

Where available, aggregate human oral exposure estimates were obtained for the chemicals based on maximally allowed chemical residue levels from food and drinking water. When the oral equivalent doses for the *in vitro* assays were compared with highest estimated human oral exposures for these chemicals, the exposures were generally well below the range of estimated oral equivalent doses. This margin was present even though comparisons were made between the most highly potentially exposed human subpopulation and lowest fifth percentile of oral equivalent doses for the simulated human cohort. Only two chemicals, pyrithiobac-sodium and triclosan, had oral equivalent doses that overlapped estimated human oral exposures. These two chemicals would not have been identified from the *in vitro* AC_{50} and LEC values, the oral equivalent doses, or the exposure estimates alone. In general, it will be necessary to have hazard information based on *in vitro* concentration-response coupled with exposure information to develop robust testing priorities. The linkage of hazard identification, dose-response, and exposure are the cornerstones of risk assessment (NRC, 1983).

The PK approaches presented in this study have the potential to extend the hazard identification paradigm toward the use of *in vitro* data in a risk assessment context. Nonetheless, there are important qualifications that are inherent in the application of these approaches. First, assumptions were made in the *in vitro*-to-*in vivo* extrapolation modeling regarding the absorption and excretion of the chemicals. Each chemical was assumed to be completely absorbed, and excretion was limited to the renal route with a rate equivalent to glomerular filtration multiplied by the amount of unbound chemical in the plasma. These assumptions should 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 steady-state plasma concentration. An exception to this would be chemicals that are subject to active renal resorption, which would lead to a higher steady-state plasma concentration at a given dose. Second, although the AC_{50} value has become a standard way to compare potencies of chemicals in *in vitro* pharmacology and toxicology studies, they may not be the best metric for prioritization or estimating toxicological risk based on well-designed *in vitro* tests. Other statistically robust methods, such as benchmark dose analysis (Crump, 1995), may be needed to provide the minimum concentration required to observe a biological effect above that seen in the vehicle-treated controls. Third, the steady-state blood concentration may not always be a good surrogate for the concentration of the chemical in the media of the *in vitro* assay. The protein and lipid composition of the media, binding to plastic as well as other variables, may affect the free concentration of the chemical and limit the ability to strictly relate blood and media concentrations (Blaauboer, 2010). Fourth, as stated in the original article outlining the use of the *in vitro* assays in toxicity testing (Judson *et al.*, 2010), the limited biotransformation capacity in many of the assays limits the ability to assess the activity of potential metabolites. Similarly, the PKs of only the parent chemical were evaluated in this study. To evaluate the biological activity and dosimetry of all the corresponding metabolites would require additional studies that would limit the throughput of the approach. Finally, the use of sensitive *in vitro* assays, such as those used in ToxCast, to accurately predict adverse responses in humans is still an open question and the subject of ongoing investigation (Judson *et al.*, 2010). Activation of these *in vitro* endpoints does not necessarily represent an adverse biological response but should be regarded as a measure of possible biological perturbations. There has been ongoing debate within the toxicological community regarding the difference between adverse and adaptive responses, and the debate is likely to continue despite the development of more sophisticated technologies and increased data collection. Further analysis is currently underway within the ToxCast program to develop predictive signatures consisting of different *in vitro* assays to predict *in vivo* responses. These assays would constitute prediction of the likelihood of an adverse response for which an oral equivalent dose could be estimated.

This study has implications for current efforts to overhaul existing chemical testing methods and utilize relatively inexpensive high-throughput *in vitro* assays to address the disparity in the number of tested and untested chemicals in commerce. From an economic and animal welfare perspective, the use of only *in vitro* AC_{50} and LEC values for prioritization could identify putative hazards that would lead to unnecessary animal testing for compounds that do not have relevant exposures. From a public safety perspective, the use of only *in vitro* AC_{50} and LEC values for prioritization could under- or overestimate the risks associated with other chemicals because of aspects of biology that are either not captured *in vitro* or not modeled *in silico*. The integration of dosimetry and human exposure information with the results from high-throughput toxicity screening efforts provides a better basis for informed decisions on chemical testing priorities and regulatory attention (Blaauboer, 2010; Cohen Hubal *et al.*, 2010). In addition, these tools are key components of human-based toxicity pathway proposals that would move beyond prioritization to a new paradigm for toxicity testing and risk assessment (NRC, 2007).

SUPPLEMENTARY DATA

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

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