Incorporating High-Throughput Exposure Predictions With Dosimetry-Adjusted In Vitro Bioactivity to Inform Chemical Toxicity Testing

Barbara A. Wetmore,*1 John F. Wambaugh,† Brittany Allen,* Stephen S. Ferguson,‡,2 Mark A. Sochaski,* R. Woodrow Setzer,† Keith A. Houck,† Cory L. Strope,* Katherine Cantwell,* Richard S. Judson,† Edward LeCluyse,* Harvey J. Clewell,* Russell S. Thomas,*†,3 and Melvin E. Andersen*

*The Hamner Institutes for Health Sciences, Institute for Chemical Safety Sciences, Research Triangle Park, North Carolina 27709-2137; †United States Environmental Protection Agency, Office of Research and Development, National Center for Computational Toxicology, Research Triangle Park, North Carolina 27711; and ‡Life Technologies, ADME/Tox Division of the Primary and Stem Cell Systems Business Unit, Durham, North Carolina 27703

1To whom correspondence should be addressed at The Hamner Institutes for Health Sciences, Institute for Chemical Safety Sciences, PO Box 12137, 6 Davis Drive, Research Triangle Park, NC 27709 Fax: (919) 558-1300. E-mail: bwetmore@thehamster.org.
2Present address: National Institute of Environmental Health Sciences, National Toxicology Program, Research Triangle Park, NC 27711.
3Present address: United States Environmental Protection Agency, Office of Research and Development, National Center for Computational Toxicology, Research Triangle Park, NC 27711.

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ABSTRACT

We previously integrated dosimetry and exposure with high-throughput screening (HTS) to enhance the utility of ToxCast HTS data by translating in vitro bioactivity concentrations to oral equivalent doses (OEDs) required to achieve these levels internally. These OEDs were compared against regulatory exposure estimates, providing an activity-to-exposure ratio (AER) useful for a risk-based ranking strategy. As ToxCast efforts expand (ie, Phase II) beyond food-use pesticides toward a wider chemical domain that lacks exposure and toxicity information, prediction tools become increasingly important. In this study, in vitro hepatic clearance and plasma protein binding were measured to estimate OEDs for a subset of Phase II chemicals. OEDs were compared against high-throughput (HT) exposure predictions generated using probabilistic modeling and Bayesian approaches generated by the U.S. Environmental Protection Agency (EPA) ExpoCast program. This approach incorporated chemical-specific use and national production volume data with biomonitoring data to inform the exposure predictions. This HT exposure modeling approach provided predictions for all Phase II chemicals. OEDs were compared against high-throughput (HT) exposure predictions generated using probabilistic modeling and Bayesian approaches generated by the U.S. Environmental Protection Agency (EPA) ExpoCast program. This approach incorporated chemical-specific use and national production volume data with biomonitoring data to inform the exposure predictions. This HT exposure modeling approach provided predictions for all Phase II chemicals assessed in this study whereas estimates from regulatory sources were available for only 7% of chemicals. Of the 163 chemicals assessed in this study, 3 or 13 chemicals possessed AERs <1 or <100, respectively. Diverse bioactivities across a range of assays and concentrations were also noted across the wider chemical space surveyed. The availability of HT exposure estimation and bioactivity screening tools provides an opportunity to incorporate a risk-based strategy for use in testing prioritization.

Key words: predictive toxicology; ToxCast; in vitro-in vivo extrapolation; dosimetry; exposure assessment
Since the release of the NRC’s (2007) “Toxicity Testing in the 21st Century,” governmental, academic, and industry researchers have dedicated significant research resources to generate data, make it publically accessible, and determine the utility of high-throughput (HT) and in vitro tools in chemical toxicity testing. The U.S. Tox21 and ToxCast research programs have leveraged HT assays developed for the pharmaceutical industry to characterize biological activities and forecast effects that may be elicited following chemical exposure (Attene-Ramos et al., 2013; Dix et al., 2007; Judson et al., 2010; Kavlock et al., 2012). Additional efforts are underway to assess in vitro strategies that identify toxicity pathways most relevant for industrial chemicals and to determine the concentrations at which perturbations and adverse effects are likely to arise (Adelleye et al., 2015; Landesmann et al., 2013; Mennecozzi, 2012). These in vitro testing efforts are complemented by bioinformatic and data visualization tools that have emerged from high-throughput screening (HTS) and genomics research efforts (McMullen et al., 2014; Pastrello et al., 2014; Pleil et al., 2011; Reif et al., 2013). Although the maturation and refinement of these in vitro and HT testing tools are promising for EPA decision-making, these tools are limited to providing hazard-based assessments. The lack of exposure information makes use in risk-based assessments difficult.

To be useful in the emerging next generation of risk science (Krewski et al., 2014), dosimetry-adjusted in vitro bioactivity data (Rotroff et al., 2010b; Wetmore et al., 2012) will need to be framed in the context of human exposure. This context will inform whether concentrations eliciting activity in the bioassays will be encountered in relevant in vivo chemical exposure scenarios. Development of a HT exposure estimation strategy will complement data obtained from HT testing programs such as ToxCast. Published HT exposure modeling tools have been largely limited to assessing chemical fate and transport from far field sources (Arnott et al., 2006; Rosenbaum, 2008). Although an important first step, HT modeling tools that capture both far- and near-field sources of chemical exposure are necessary to provide a more realistic estimate of daily human exposures.

In this report, we describe the first attempt to incorporate HT chemical toxicity testing data with HT predictions of exposure to provide a rapid, risk-based prioritization approach. In vitro assays measuring hepatic clearance and plasma protein binding conducted on ToxCast Phase II chemicals parameterize a pharmacokinetic (PK) model based upon in vitro–in vivo extrapolation (IVIVE). This model was used to predict the chemical steady-state concentrations (Css) in plasma resulting from repeated daily exposure (Rotroff et al., 2010b; Wetmore et al., 2012). Reverse dosimetry (Tan et al., 2007) tools were then used to estimate the oral equivalent dose (OED), in mg/kg/day, required to achieve blood Css levels identical to the activity concentrations (eg, ACss) in the ToxCast assays. These OEDs were then compared against exposures from a probabilistic prediction tool developed by the USEPA ExpoCast program. This tool utilizes chemical-specific use and production data that have been found to correlate with chemical exposures inferred from urinary analyte (exposure biomonitoring) data from the Center for Disease Control’s (CDC’s) National Health and Nutrition Examination Survey (NHANES). The NHANES characterizes central tendencies (ie, geometric means) of chemical exposures for populations in the United States (Calafat, 2012). Bayesian modeling is used to both account for unknown information that is needed to predict exposures while also quantifying the uncertainty of the predicted geometric means (Wambaugh et al., 2014). Comparison of the OEDs to these exposure predictions, both expressed in mg/kg/day, provides a useful first-order approximation of activity-to-exposure ratios (AERs)—in essence a margin of exposure (MOE)—that can help shift from a hazard-centric approach toward a more risk-based strategy that can inform prioritization strategies (Thomas et al., 2013).

**MATERIALS AND METHODS**

Chemical selection and stock preparation. The 178 ToxCast Phase II chemicals (http://www.epa.gov/nctc/toxcast/chemicals.html) [last accessed August 20, 2015] analyzed in this study were selected based on the existence of an analytical chemistry detection method and the availability of human exposure data. Compounds for the plasma protein binding and metabolic stability assays were obtained from Compound Focus, Inc (Evotec, South San Francisco, California) in neat form. Dimethyl sulfoxide (DMSO) stock solutions were prepared from the neat chemicals to generate the analytical calibration curves and for use in the assays. All stock solutions were stored at < −70°C. Specific vendor and vendor-supplied purity information for each chemical is provided as Supplementary material (Supplementary Table S1).

Plasma protein binding assay. Plasma protein binding was measured for each chemical using either the rapid equilibrium dialysis (RED) method as described previously (Rotroff et al., 2010b; Waters et al., 2008; Wetmore et al., 2012) or ultrafiltration as described later. The human plasma used in the assay was obtained from healthy, consented, paid donors at a U.S. Food and Drug Administration-licensed and inspected donor center (HMPLEDTA2; Bioreclamation, Inc, Westbury, New York). The plasma was pooled from 5 male (37, 22, 27, 36, and 21 years old) and 5 female (30, 40, 47, 55, and 54 years old) adults and stored at < −70°C until use.

Determination of plasma protein binding by ultrafiltration was conducted on a subset of chemicals for which equilibration dialysis resulted in unbound values > 100%. This phenomenon has been observed with a subset of ToxCast industrial chemicals (eg, plasticizers, phthalates) and is believed to occur due to binding and/or interactions with dialysis plate components (data not shown). Briefly, plasma was thawed to room temperature and, if necessary, pH adjusted to 7.4. DMSO stocks of chemicals (200X) were added to plasma to achieve a final concentration of 10 μM. Samples were vortexed and incubated at 37°C in a water bath in polypropylene tubes prior to centrifugation in a Centrifree ultrafiltration device (Millipore Cat No. 4104, Billerica, Massachusetts) at 2000 × g for 20 min at 37°C. Ultrafiltrates were collected for analysis. This procedure ensured that the ultrafiltrate did not exceed 40% of the initial volume and minimized dissociation of bound compound due to removal of free compound (Whitlam and Brown, 1981). Nonspecific binding (NSB) was measured in a similar manner, with chemical stocks added to phosphate-buffered saline, pH 7.4 to achieve a final concentration of 10 μM, incubated at 37°C, and aliquots collected from both the preCentrifree device incubation and the post-centrifugation ultrafiltrate. All samples were run in triplicate and stored at < −70°C prior to analysis.

Metabolic clearance assay. Hepatic clearance was measured using the substrate depletion method (Wetmore et al., 2012). Chemicals at 2 concentrations (1 and 10 μM) were incubated over a 240-min period with pooled cryopreserved primary human hepatocytes (Life Technologies; Durham, North Carolina). The pool of cryopreserved hepatocytes was...
Chemical analysis by liquid chromatography with mass spectrometric detection. Samples from the metabolic stability assay (quenched 1:1:6, Plasma/PBS:Acetonitrile) were thawed at room temperature, vortexed briefly, and centrifuged at 12 000 × g for 4 min. All plasma samples were prepared as outlined earlier for the 1 μM metabolic stability assay samples (ie, 1:4 dilution). Detailed chromatographic separation protocols along with mass spectrometric (MS) information for all compounds analyzed by liquid chromatography (LC)/MS are provided in Supplementary Tables S2A–C.

Chemical analysis by GC with electron capture detection. Both metabolic stability assay samples and protein binding samples were obtained in the same dilutions described in the HPLC/MS methods earlier. All samples were thawed at room temperature, vortexed briefly, and centrifuged at 4500 × g for 5 min. Prior to liquid extraction, samples were spiked with a solution containing a known amount of internal standard and diluted 3:2 with a saturated NaCl solution for the metabolic stability assay samples and 2:3 for the protein binding samples. Samples underwent 1 hexane extraction (150 μl, nanograde quality), were vortexed briefly, allowed to equilibrate for 30 min, and centrifuged at 1300 × g for 2 min. The hexane layers were collected and transferred to silanized glass inserts prior to analysis using an Agilent 6890 gas chromatograph with a model 5973 MS (Agilent Technologies) in either electron impact mode or negative chemical ionization mode. Calibration standards were prepared on the same day as sample analysis and in a matrix identical to the samples. Sample data were collected in selective ion-monitoring (SIM) mode. Specific chromatographic separation details and instrumental parameters for each analyte are provided as Supplementary material (Supplementary Table S2D).

Chemical analysis by HPLC with fluorescence detection (HPLC/FLD). 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 ultraviolet/fluorescent detectors (Agilent Technologies) without any additional sample work-up. Chromatographic separation details, fluorescence settings, and analyte elution times are described in the Supplementary materials section (Supplementary Table S2E).

Plasma protein binding data analysis. To calculate the fraction of unbound chemical in the plasma (F_u) from equilibrium dialysis data, the concentration of the test compound in the phosphate buffered saline (PBS) chamber was divided by the mean concentration in the matched plasma sample. Values derived for the 3 replicates were then averaged to determine a mean F_u. A minimum measurable F_u was set to 0.005. This value was estimated based on 2 SD over the minimum amount of binding detected in...
a previous study (Waters et al., 2008) and previous experience with the RED method (Rotroff et al., 2010b; Wetmore et al., 2012). If the concentration of the chemical in the free fraction was below, this value or below the analytical limits of detection, a default $F_u$ of 0.005 was assumed.

To calculate $F_u$, from the ultrafiltration data, the concentration of the test compound in the plasma ultrafiltrate was divided by the concentration in the precentrifugation sample for each replicate. The average mean percent unbound was calculated for the 3 replicates run. Mean percent unbound values were calculated in the same way for the NSB samples. Chemicals with NSB values exceeding 5% were excluded from further analyses. The plasma protein binding data are provided in Supplementary Table S3A.

Metabolic clearance data analysis. Hepatic metabolic clearance ($Cl_{\text{in vitro}}$) was determined following linear regression analysis of data measuring the loss of chemical over time (Rotroff et al., 2010b; Wetmore et al., 2012). Clearance was normalized to cell number ($\mu$l/min $\times 10^6$ cells). The concentration data at each time point for each chemical and the linear regression results are provided as Supplementary Table S3B.

A NSB of a chemical that occurs may limit the amount of chemical available for clearance in an in vitro system (Hallifax et al., 2010). Estimating clearance through loss of parent compound as done with the substrate depletion approach may lead to an underestimation of clearance for highly bound compounds. Although a nonspecifically bound chemical cannot be metabolized, it is still present in the incubation mixture and pounds. Although a nonspecifically bound chemical cannot be to an underestimation of clearance for highly bound compound as done with the substrate depletion approach may lead. The basic equation used to calculate static clearance and nonmetabolic renal clearance:

$$Cl_{\text{in vitro}} = \frac{Cl_{\text{intH}}}{F_{\text{ub}} + Cl_{\text{meth}}}$$

where $Cl_{\text{intH}}$ is the intrinsic hepatic clearance, $Cl_{\text{meth}}$ is the metabolic clearance, and $F_{\text{ub}}$ is the fraction unbound.

Calculation and statistical presentation of OED data. As previously described, the in vitro $AC_{50}$ (concentration at 50% of maximum activity) or lowest effective concentration (LEC) values were assumed to be functionally equivalent to the $C_{50}$ values in terms of biological activity (Rotroff et al., 2010b; Wetmore et al., 2012). Using reverse dosimetry (Tan et al., 2007), the median, 5th, and 95th percentiles for the $C_{50}$ were used as conversion factors to generate OEDs according to the following formula:

$$OED \left( \frac{\text{mg/kg}}{\text{d}} \right) = \text{ToxCast } AC_{50}\text{ or } \text{LEC} \left( \mu\text{M} \right) \frac{(1 \text{ mg/kg)/day}}{C_{50} \left( \mu\text{M} \right)}$$

In the equation, the OED is linearly related to the in vitro $AC_{50}$ or LEC and inversely related to $C_{50}$. This equation is valid only for first-order metabolism that is expected at ambient exposure levels. An OED was generated for each chemical and each $AC_{50}$ or LEC value across all of the in vitro assay endpoints.

Box and whisker plots were used to visualize the OEDs for each chemical. In each figure, the 95th percentile of the $C_{50}$ was used in the figures to provide a conservative estimate of the OEDs. The median OED for each chemical was displayed as a horizontal line and the ends of the boxes represent 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 or 75th percentiles, respectively (Tukey, 1977). In those instances where the minimum or maximum value for that chemical 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 PK modeling. Published human in vivo PK data from which $C_{50}$ values could be derived were available for 16 of the 178 chemicals analyzed. These data characterized the observed total clearance from the body including hepatic metabolism and glomerular filtration as well as other PK pathways present in vivo. To assess the predictivity of our IVIVE model, $C_{50}$ values were calculated using the measured in vivo values,
assumed a daily oral dose of 1 mg/kg/day. These values were then compared against the IVIVE-derived values obtained using the in vitro clearance rate derived using 1 μM chemical concentration. In addition, Caco-2 data was incorporated into the IVIVE to assess the impact of the assumption of 100% absorption on the prediction of \( C_{\text{\text{ss}}} \). Further, for those chemicals that displayed no measurable clearance in the hepatocyte suspensions, plated hepatocytes were employed to measure clearance via substrate depletion over 48 h.

In vitro bioactivity data. To date, ToxCast bioactivity data includes measured bioactivity screening data across over 1000 compounds against a set of approximately 780 in vitro assay endpoints. Data from the December, 2014 release were downloaded from the ToxCast website (http://epa.gov/ncct/toxcast/data.html) [last accessed August 20, 2015]. 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-assay combination was run in concentration response and an AC_{50} or LEC value was calculated, if applicable, depending on the range of the concentration response data. The data utilized include outputs from a new data processing pipeline http://epa.gov/ncct/toxcast/files/MySQL-20Database/Pipeline_Overview.pdf. In addition to revised AC_{50} outputs, data quality flags have been incorporated to alert users to experimental issues that may confound data interpretation. The chemical-assay hits of relevance for this study were reviewed for presence of a potential data quality issue, indicated by 1 of 17 flags that encompass issues across all of the ToxCast assay platforms (for more information, visit http://epa.gov/ncct/toxcast/data.html). Given that many of the flags are platform-specific and this assessment was comprehensive, spanning all chemical-assay hits across all of the technologies but with a focus on the most potent AC_{50} for AER derivation, any of these hits tagged with any flag was removed from the assessment. Although not the most conservative approach, this method using the higher confidence in vitro bioactivity results was selected for an illustrative example. The original list of 8963 chemical-assay hits across the 178 chemicals was thus filtered down to a list of 4582 hits across 163 of the chemicals.

Several peer reviewed publications utilizing the bioassay data from Phase I (Houck et al., 2009; Huang et al., 2011; Judson et al., 2010; Kleinstreuer et al., 2014; Knight et al., 2009; Knudsen et al., 2011; Martin et al., 2010; Rotroff et al., 2010a, 2013) and 2 from Phase II (Kleinstreuer et al., 2014; Sipes et al., 2013) are available and provide additional information. A detailed description of the chemicals screened, assays used and details related to the new pipeline outputs can be found at the USEPA download site (http://epa.gov/ncct/toxcast/data.html).

Exposure prediction methods. A probabilistic exposure modeling approach was employed, as detailed in (Wambaugh et al., 2014). Briefly, subject-specific NHANES urinary analyte data were collected and analyzed in a reverse PK approach that used a parent-to-analyte mapping to infer parent compound exposure for 106 chemicals. Because there were multiple combinations of parent chemical exposures that were consistent with the analyte data, a range of possible combinations of inferred parent chemical exposures was analyzed. Chemicals were assigned indicator variables (with value 1 or 0 corresponding to yes or no) indicating evidence for use of that chemical within broad use categories (eg, consumer use, pesticide active) based on listings in U.S. EPA’s ACToR (Aggregated Computational Toxicology Resource) database (Dionisio et al., 2015). Chemicals were further characterized using physico-chemical properties and national production volume data. These simple chemical descriptors were chosen because they were available for thousands of chemicals.

To identify those factors that most correlated with the range of inferred chemical exposures, Wambaugh et al. (2014) assumed a linear model in which the logarithm of inferred parent exposure depended on an average value and, potentially, some factors among production volume, chemical use indicator variables, and physico-chemical properties. Each of the factors in the linear model were scaled and centered and multiplied by a weight that indicated the relative importance to the model. The selection of the most predictive factors was performed using the method of best subsets to estimate regression weights best subset selection was performed using complete enumeration of factor combinations (Morgan and Tatar, 1972). This process was repeated across the range of possible chemical exposure scenarios to identify the minimum number of factors required to build a parsimonious model, using the average Akaike information criterion (AIC) (Akaike, 1974) across the scenarios. A 5-factor model was suggested by AIC. The frequency of occurrence of the factors among the best subset size was used to determine the optimal model.

Using the factors identified by the best subsets analysis a second Bayesian regression was performed to jointly infer the regression coefficients, stoichiometric relationships among metabolites, and parent exposures from the NHANES urinary data. This joint Bayesian analysis was performed separately for the entire NHANES samples (roughly 2000 individuals per chemical) and subsets of that sample corresponding to 9 demographic groups and life stages, including: children 6–11 years of age, children 12–19 years of age, adults 20–65 years of age, females adults (6–85), males (6–85), adults older than 65 years, females of child-bearing age (16–49), and adults older than 65 years of age (65–85). Also assessed were adults (mixed gender, age range) with a body mass index (BMI) < 30 and a BMI > 30. A calibrated model based on the same 5 factors was found to be predictive across all groups.

The Wambaugh et al. (2014) calibrated model explained roughly 50% of the chemical-to-chemical variance within the biomonitoring data. The remaining unexplained variance served as an empirical estimate of the uncertainty in the predictions, due to assumptions of the modeling, measurement limitations of the data, quality issues in the chemical descriptors, and any other factor not taken into account by the modeling analysis of the 106 chemicals that could be inferred from NHANES urine analytes. Both the calibrated model and empirical estimate of the uncertainty were extrapolated to predict exposure for chemicals without biomonitoring data. The Bayesian analysis was used to predict geometric mean population exposures with 95% credible intervals around the mean estimates. The model weights and chemical-specific predictions and descriptors are given in Wambaugh et al. (2014).

RESULTS

Evaluation of PK Modeling

Of the 16 chemicals for which \( C_{\text{\text{ss}}} \) values were derived from published human in vivo PK data, 11 were within 10-fold of the IVIVE-derived \( C_{\text{\text{ss}}} \) predictions (Table 1). When the IVIVE was
refined through the incorporation of Caco-2 data (to replace our assumption of 100% absorption with experimental data) and of revised clearance data using plated hepatocytes, predictions for 12 of the 16 compounds came within 6-fold of the IVIVE values. The 4 chemicals that performed poorly were all overpredicted: chlorpyrifos (12-fold), coumarin (87- to 173-fold) flutamide (142- to 160-fold), and lovastatin (20- to 45-fold). Although better C_{ss} values derived via IVIVE modeling, assuming an oral administration of 1 mg/kg/day across the 178 Phase II chemicals, revealed a median C_{ss} value of 0.94 μM, with approximately 80% of the chemicals possessing values < 10 μM (Fig. 1B). Moreover, the upper 95th percentile was 230 μM, with approximately 7% of the chemicals possessing a C_{ss} > 200 μM.

**Influence of C_{ss} on In Vitro Bioactivities**

To demonstrate the impact of incorporating chemical steady-state behavior on in vitro bioactivity values, Table 2 displays the range of OEDs that result across 14 chemicals (with hits listed across 18 assay endpoints) that exhibited bioactivity at an AC_{50} value of 1 μM. The minimum and maximum OEDs ranged from 0.002 (dinoseb) to 51 mg/kg/day (butylparaben), spanning over 4 orders of magnitude (25 000-fold). OEDs for 9 of the 18 chemicals were within 5-fold of each other, with values ranging from 0.31 to 1.47 mg/kg/day.

#### Distribution Analysis of AC_{50} and C_{ss} Values

Distribution analysis of the minimum AC_{50} values derived for each chemical across all assay technologies revealed that the minimum value was 7.4E-05 μM for diethylstilbesterol. The median was 1.6 μM, with the lower 5th, 10th, and 25th percentiles at 0.004, 0.012, and 0.259 μM, respectively (Fig. 1A). The highest minimum AC_{50} value was 91.4 μM for 1,3-diisopropylbenzene. Assessment of the C_{ss} values derived via IVIVE modeling, assuming an oral administration of 1 mg/kg/day and spanning over 4 orders of magnitude (25 000-fold). OEDs for 9 of the 18 chemicals were within 5-fold of each other, with values ranging from 0.31 to 1.47 mg/kg/day.

#### TABLE 1. Comparison of IVIVE C_{ss} Predictions with Published In Vivo-Derived Values

| Chemical | C_{ss} Values (μM) | Fold Difference | Key to Prediction Improvement | References for In Vivo Calculations |
|----------|------------------|----------------|-------------------------------|-------------------------------------|
|          | In Vivo | IVIVE | Caco-2 | HT | Key to Prediction Improvement | References for In Vivo Calculations |
| Acetaminophen | 1.1  | 0.52 | 0.57 | — | 0.5 | 0.5 | Within 2-fold | (Critchley et al., 2005; Gelotte et al., 2007; Rostami-Hodjegan et al., 2002) |
| 2-chloro-2-deoxyadenosine | 0.28 | 1.36 | 0.58 | 0.31 | 4.9 | 1.1 | Within 5-fold | (Lindermalm et al., 2005) |
| 5,5’-diphenylhydantoin | 4.92 | 1.59 | 1.59 | — | 0.3 | 0.4 | Within 4-fold | (Brien et al., 1995) |
| 6-propyl-2-thiouracil | 1.1  | 1.58 | 1.80 | — | 1.3 | 1.5 | Within 2-fold | (Giles et al., 1981; Kabanda et al., 1996) |
| Candoxatril | 0.023 | 0.18 | 0.14 | — | 7.8 | 6.1 | Within 6-fold | (Kaye et al., 1997) |
| Chlorpyrifos | 0.022 | 0.24 | 0.27 | — | 10.9 | 12.3 | Unknown | (Nolan, 1984; 371) |
| Coumarin | 0.01-0.02 | 13.63 | 15.40 | 1.73 | 681–1363 | 87–173 | Plated hepatocytes | (Lamiable et al., 1993; Mielke et al., 2011) |
| Diphenhydramine HCl | 0.11–0.16 | 3.18 | 3.57 | 0.66 | 20–29 | 4–6 | Plated hepatocytes | (Albert et al., 1975; Blyden et al., 1986; Luna et al., 1989; Toothaker et al., 2000) |
| Flutamide | 0.004–0.005 | 0.57 | 0.64 | — | 142 | 160 | Inclusion of intestinal metabolism | (Anjum et al., 1999; Doser et al., 1997; Radwanski et al., 1989) |
| Haloperidol | 0.126 | 0.07 | 0.08 | — | 1.8 | 1.6 | Within 2-fold | (Yasui-Furukori et al., 2002) |
| Lovastatin | 0.004–0.009 | 0.16 | 0.18 | — | 18–40 | 20–45 | Unknown | (Bramer et al., 1999; Kothare et al., 2007; Mignini et al., 2008) |
| PK 11195 | 0.14 | 0.58 | 0.66 | — | 4.1 | 4.7 | Within 5-fold | (Ferry et al., 1989) |
| Sulfasalazine | 0.2–1.8 | 11.6 | 2.5 | — | 7–48 | 1–10 | Caco-2 | (Adkison et al., 2010; Gu et al., 2011; Ma et al., 2009) |
| Triamcinolone | 0.05–0.29 | 0.22 | 0.11 | — | 0.8–4.4 | 0.4–2.2 | Within 5-fold | (Argenti et al., 2000; Derendorf et al., 1995; Hochhaus et al., 1990) |
| Volinanserin | 0.04 | 0.03 | 0.03 | — | 3.8 | 4.3 | Within 4-fold | (Andree et al., 1998) |
| Zamifenacin | 2.86 | 0.57 | 0.64 | — | 0.2 | 0.2 | Within 5-fold | (Beaumont et al., 1996) |

*Values from 2 studies were 1.05 and 1.12; for purposes of this work, 1.1 μM was used as comparator.
FIG. 1. Distribution and summary statistics of activity concentration (AC50) and Css values. A, The minimum AC50 values derived across all technologies for each chemical underwent distribution analysis and were binned across 7 concentration ranges to display the number of values (bar graph) and cumulative frequency (line graph) across the relevant range, with the summary statistics provided. B, The 95th percentile Css values (μM) was predicted using the hepatic chemical clearance rate measured at 1 μM across a population of 10,000 individuals (using Monte Carlo simulation, assuming a unit dose rate of 1 mg/kg/day; see Materials and Methods) were binned and displayed in a manner similar to A. Values are provided from highest to lowest as a higher predicted Css may indicate a higher chemical exposure. Summary statistics are also provided.

TABLE 2. Oral Equivalent Dose Ranges for Chemicals with Identical In Vitro Potencies but Varied Steady-State Behavior

| Chemical                     | Css [μM] | Assay Endpoint                                                                 | AC50 [μM] | OED [mg/kg/day] |
|------------------------------|----------|---------------------------------------------------------------------------------|------------|-----------------|
| Dinoseb                      | 485.94   | Agonist for p53 signaling pathway in HCT-116 cells                              | 1          | 0.002           |
| Gentian violet               | 10.01    | Decreased expression of tissue matrix metalloprotease inhibitor-2 in human keratinocytes | 1          | 0.095           |
| Gentian violet               | 10.01    | Binding to muscarinic acetylcholine receptor M2                                 | 1          | 0.096           |
| Gentian violet               | 10.01    | Decreased expression of urokinase receptor in human endothelial cells           | 1          | 0.098           |
| Didecyl dimethyl ammonium chloride | 3.37    | Decreased expression of collagen type III in human primary fibroblasts         | 1          | 0.306           |
| Dieldrin                     | 2.32     | Activation of estrogen receptor response element in transfected HepG2 cells     | 1          | 0.431           |
| 2-Chloro-2′-deoxyadenosine   | 2.07     | Decreased expression of membrane protein CD40 in human endothelial cells       | 1          | 0.464           |
| 9-Phenantral                 | 2.14     | Decreased proliferation of human primary fibroblasts                            | 1          | 0.481           |
| Ethion                       | 1.40     | Activation of the phenobarbital-responsive enhancer module in transfected HepG2 cells | 1          | 0.711           |
| Pentachlorophenol            | 0.87     | Inhibition of the peroxisome proliferator-activated receptor gamma signaling pathway in HEK293 cells | 1          | 1.143           |
| o,p-DDT                      | 0.80     | Activation of estrogen receptor response element in transfected HepG2 cells     | 1          | 1.232           |
| Zamifenacin                  | 0.69     | Binding to guinea pig dopamine transporter                                     | 1          | 1.457           |
| Zamifenacin                  | 0.69     | Binding to human 5-hydroxytryptamine-7 (5HT7) receptor                         | 1          | 1.471           |
| Benz[a]anthracene            | 0.47     | Increased expression of matrix metalloprotease-1 in human primary bronchial epithelial cells | 1          | 2.053           |
| Diethylstilbesterol (DES)    | 0.46     | Inhibition of rat CYP2C19 enzymatic activity                                   | 1          | 2.151           |
| N-Phenyl-1,4-benzenediamine  | 0.33     | Decreased expression of tissue factor in human endothelial cells               | 1          | 2.927           |
| Butylparaben                 | 0.02     | Activation of estrogen receptor alpha signaling pathway in transfected HepG2 cells | 1          | 51.140          |

Css, Concentration at steady state.  
OED, oral equivalent dose.
Assessment of Exposure Predictions

The HT exposure method makes chemical-specific predictions for the geometric mean for U.S. populations. Uncertainty in the estimates is characterized by a 95% confidence. The upper 95% confidence limit of the geometric mean ranged from 9.26E-07 mg/kg/day (methyl eugenol) to a maximum of 8.46E-03 mg/kg/day (di(2-ethylhexyl)adipate). The range of the 95% confidence limits were on average 4 orders of magnitude. Comparison of the predictions for the total population against the most highly exposed (MHE) population for each chemical revealed that the MHE values were on average 2- to 3-fold higher (Supplementary Table S4). However, for the HT exposure model that was used, there were no statistically significant differences in the mean prediction by the model for the various populations. For instance, of the 163 chemicals assessed, the 2 BMI groups (BMI > 30 and BMI < 30) emerged as being the predominant MHE population for 32 and 31 chemicals, respectively (Supplementary Table S4). This finding is likely artifactual due to the relatively sensitive nature of the 95th percentile to the relative sizes of the sample populations analyzed. The third most prevalent MHE population was the 12- to 19-year-old group, for 26 chemicals.

Assessment of Dosimetry-Adjusted ToxCast Assay Activity With HT Exposure Predictions

Figure 2 displays the range of OEDs derived for each chemical across all relevant assays in a box and whisker format, superimposed with floating bars that provide HT exposure predictions (Wambaugh et al., 2013, 2014). In Figure 2, the floating bars represent the predictions across the total population, with the median assigned the lower bound value and upper 95% of the credible interval around the median assigned the upper-bound value. The red circle represents the upper 95% confidence interval for the MHE population.

Of the 178 chemicals for which hepatic clearance and plasma protein binding were successfully measured, 163 possessed at least 1 ToxCast assay in which bioactivity was observed/measurable (ie, an AC50 or LEC was estimated). HT exposure predictions were available for all 163 chemicals. AERs were calculated for each chemical by dividing the minimum OED (ie, the most potent assay for that chemical) by the upper bound of the 95% confidence interval of the geometric mean for the exposure predictions. When AERs were calculated using the upper-bound exposure predictions for the total population, 3, 6, and 13 chemicals possessed AERs < 1, 10, and 100, respectively. When AERs were calculated using the upper-bound predictions for the MHE populations, 5, 9, and 19 chemicals possessed AERs < 1, 10, and 100, respectively (Supplementary Table S4). Distribution of the AERs across the Phase II chemicals assessed in this study revealed median values of 2.04E-04 and 9.58E+03 for the total and MHE populations, respectively (Fig. 3).

Closer inspection of the twenty chemicals with the lowest AERs revealed that organofluorines and insecticides previously withdrawn from the market comprised 5 of the 12 chemicals (Table 3). Tannic acid, a plant polyphenol with food and drug uses yielded the lowest AER (MHE AER 0.017 mg/kg/day). This was derived based on an OED of 5.83E-04 mg/kg/d for a cell-free assay measuring glycogen synthase kinase 3 beta (GSK3b) activation, an enzyme involved in energy metabolism and neuronal development (Plyte et al., 1992). Of the 12 chemicals with AERs < 1, only 2—napthalene (6 hits) and organofluorine heptadecafluoroctanesulfonic acid, potassium salt (2 hits)—had bioactivities measured in more than 1 assay. A complete listing of chemicals, associated uses and specific information for all assays that yielded an AER < 10 is provided in Supplementary Table S4.

Assessment of the OED Findings

The potency of a chemical’s OED could be due to either a low ToxCast assay AC50 value (ie, potent activity), a high C50 value resulting from the IVIVE, or a combination of the 2. A subset of chemicals possessing low OEDs was more closely examined to assess the relative contribution of these 2 factors on the final values across this chemical space. Of the 11 chemicals that possessed OEDs < 1 μg/kg/day, 3 were perfluorinated compounds, 3 were insecticides which had been withdrawn from the market, 2 were pharmaceutical compounds, and 1 a plant polyphenol (Table 3). All but 2 of the chemical-assay hits possessed an

FIG. 2. Comparison of human oral equivalent doses (OEDs) and exposure predictions for 163 ToxCast Phase II chemicals. Distributions of the OEDs across approximately 700 in vitro assays for each chemical are depicted as box-and-whisker plots, presented with exposure predictions derived from (Wambaugh et al., 2014). Data are ordered from lowest to highest median assigned the lower bound value and upper 95% confidence limit around the mean. The red filled circle denotes the upper 95% confidence limit derived for the most highly exposed (MHE) population for that chemical. Arrows indicate chemicals with AERs < 1.
AC50 < 0.5 μM. Six of the eleven chemicals possessed a C50 > 200 μM—a criterion representative for the upper 10th percent of all chemicals. Three of the 6 chemicals with high C50 values were organofluorines, most of which had former uses as flame retardants.

**DISCUSSION**

To assess the utility of in vitro HTS data to predict chemical hazard to human health, the USEPA ToxCast program has evaluated libraries of chemicals in multiple phases. Phase I assessments screened and analyzed data-rich compounds, in particular food-use pesticides, for which measured physicochemical properties, in vivo hazard data, and exposure estimates were available. Knowledge of animal study-based apical responses enabled the assessment of the HTS data for their ability to identify biological pathway alterations (Houck et al., 2009; Judson et al., 2011; Knudsen et al., 2011; Rotroff et al., 2010a) and prediction of in vivo effects (Kleinstreuer et al., 2011; Martin et al., 2011; Sipes et al., 2011; Thomas et al., 2012; Wetmore et al., 2013). Efforts to incorporate chemical dosimetry with HTS data provided an in vitro context to the in vivo data, allowing an estimation of external dose required to achieve internal bioactivity-inducing concentrations (Rotroff et al., 2010b; Wetmore et al., 2013, 2012). These studies have both indicated the potential of ToxCast data as a risk-based prioritization tool (Judson et al., 2011; Kavlock et al., 2009; Krewski et al., 2014) as well as identifying its limitations (Cox et al., 2014; Thomas et al., 2012; Wetmore et al., 2013). The data and subsequent analyses have provided useful guidance as successive phases have been undertaken.

Chemicals in the Phase II library were selected to expand the chemical space addressed in Phase I and include banned and withdrawn pharmaceutical and industrial compounds along with compounds currently in commerce (Judson et al., 2009). Inclusion of pharmaceuticals for which therapeutic activities are already established—and banned chemicals with well recognized in vivo apical responses—allows an informed assessment of the bioactivities and potencies observed within the ToxCast dataset. However, only a limited number of these chemicals possess exposure information. In previous work combining HTS data with exposure (Wetmore et al., 2012), review of USEPA reregistration eligibility documents and data collected by the CDC NHANES effort provided exposure data for over 80% of the ToxCast Phase I chemicals. When applied to the Phase II chemicals assessed in the current study, data were available for many fewer compounds, only 7%.

We addressed this in this study by employing a probabilistic modeling approach to approximate exposures in a HT manner (Wambaugh et al., 2014). Even with the 4 order of magnitude span of the 95% credible interval around the geometric mean exposure predictions (Fig. 2), the ability to compare the upper-bound predictions against dosimetry-adjusted bioactivities provides a needed, risk-based strategy that can be applied in prioritization strategies. Further, as refined exposure modeling strategies emerge, their values could be readily incorporated with in vitro data to either refine lower tier assessments or lay the groundwork for strategies to be applied in higher tiers that require more data.

Review of the Phase II chemical AER findings provides insight into future priorities in exposure modeling efforts. The frequency of AERs < 1 derived in this assessment were significantly less than if predictions from an earlier version of this modeling approach (Wambaugh et al., 2013) were employed (data not shown). This decrease is due in large part to the ability of the second model to explain 50% of the variability after assessment across multiple chemical product and use descriptors as opposed to 20% for a model based on far-field fate and transport models (Wambaugh et al., 2013). Recent—and future—efforts that increase availability of chemical use and product formulation information should help significantly in refining near-field modeling tools and reducing uncertainty around the estimates to provide more accurate exposure predictions (Dionisio et al., 2015; Goldsmith et al., 2014). It should be noted that an AER cutoff of 1 is used primarily for illustrative purposes. Given that the upper-bound exposure predictions reflect the upper 95th percent confidence limit around the geometric mean, these values do not reflect an approximation of exposures to a sensitive population. Given this, a higher AER cutoff (e.g., 100) may be more appropriate to consider in such strategies.

Phase II AER assessment also outlined important considerations related to HTS data interpretation. For instance, for all but 1 of the 7 chemicals flagged using the 2014 exposure model, only 1

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**FIG. 3.** AER distribution across the ToxCast Phase II chemicals assessed. Histograms and cumulative percent data (line graph) are displayed to capture the AER distribution across the chemicals analyzed for the total population (A) and the MHE population (B). AERs are calculated by dividing the minimum chemical OED by the upper 95% confidence limit around the mean exposure prediction (see Materials and Methods). The bar representing chemicals with AERs < 1 derived in this assessment were significantly less than if predictions from an earlier version of this modeling approach (Wambaugh et al., 2013) were employed (data not shown). This decrease is due in large part to the ability of the second model to explain 50% of the variability after assessment across multiple chemical product and use descriptors as opposed to 20% for a model based on far-field fate and transport models (Wambaugh et al., 2013). Recent—and future—efforts that increase availability of chemical use and product formulation information should help significantly in refining near-field modeling tools and reducing uncertainty around the estimates to provide more accurate exposure predictions (Dionisio et al., 2015; Goldsmith et al., 2014). It should be noted that an AER cutoff of 1 is used primarily for illustrative purposes. Given that the upper-bound exposure predictions reflect the upper 95th percent confidence limit around the geometric mean, these values do not reflect an approximation of exposures to a sensitive population. Given this, a higher AER cutoff (e.g., 100) may be more appropriate to consider in such strategies.

Phase II AER assessment also outlined important considerations related to HTS data interpretation. For instance, for all but 1 of the 7 chemicals flagged using the 2014 exposure model, only 1...
or 2 assay hits per chemical resulted in an AER < 1. The ToxCast assays were originally selected from those that were commercially available and in use by the pharmaceutical industry and, as such, the bioactivities interrogated in ToxCast focus primarily on therapeutic or receptor-mediated events. Consequently, closer examination of specific hits is warranted to differentiate biologic perturbations from measures of adversity. Importantly, HTS hits for certain pharmaceuticals in this list were consistent with their therapeutic target (Supplementary Table S4).

Comparison of the IVIVE-based predictions against in vivo data revealed that this simplified IVIVE strategy did reasonably well in predicting in vivo PK behavior: 12 of the 16 chemicals assessed coming within 10-fold of the predictions (Table 1). For the 4 that exceeded 10-fold, the \( C_{S0} \) values were all overpredicted. Three chemicals were underpredicted, but these were within 2- to 5-fold of the in vivo values. Flutamide, an antiandro

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\text{TABLE 3. Use and Assay Information for Chemicals with the 20 Lowest Activity:Exposure Ratios}
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| Chemical | Description/Use | No. Hits Where MHE* AER\(^b\) < 100 | \( AC_{50} (\mu M) ^c \) | Oral Equivalent\(^c\) (mg/kg/day) | Exposure Total (MHE) (mg/kg/day) | AER (MHE AER) |
|----------|-----------------|------------------------------------|-----------------|-----------------|-----------------|-----------------|
| Tannic acid | Plant polyphenol; food, drug uses; mordant during dyeing process | 5 | 0.0002 | 5.83E-04 | 1.35E-02 (3.36E-02) | 0.043 (0.02) |
| Triphenyl phosphate | Plasticizer; fire retardant | 3 | 0.0006 | 7.66E-04 | 6.57E-03 (1.41E-02) | 0.117 (0.054) |
| Heptadecafluoroctanesulfonic acid potassium salt | Organofluorine | 12 | 0.013 | 5.99E-05 | 3.21E-04 (8.72E-04) | 0.187 (0.069) |
| Mirex | Banned organochlorine insecticide | 3 | 0.01144 | 1.61E-04 | 1.55E-04 (3.13E-04) | 1.040 (0.516) |
| Ammonium perfluorooctanoate | Organofluorine | 9 | 0.20182 | 7.48E-04 | 3.24E-04 (1.09E-03) | 2.310 (0.684) |
| Tributyl phosphate | Solvent; plasticizer | 3 | 1.28 | 2.04E-02 | 4.03E-03 (6.60E-03) | 5.05 (3.09) |
| Potassium perfluorohexanesulfonate | Organofluorine | 2 | 0.0825 | 3.09E-04 | 3.09E-05 (7.27E-05) | 10.02 (4.26) |
| Dioctyl phthalate | Plasticizer | 6 | 4.88 | 7.62E-02 | 7.49E-03 (1.34E-02) | 10.18 (5.68) |
| DES | Nonsteroidal estrogen | 6 | 0.000074 | 1.61E-04 | 1.49E-05 (2.84E-05) | 10.82 (5.68) |
| Diphenhydramine hydrochloride | Antihistamine drug | 2 | 0.0238 | 4.91E-03 | 1.95E-04 (4.27E-04) | 25.21 (11.51) |
| Dinoseb | Herbicide | 6 | 0.35 | 7.20E-04 | 1.76E-05 (2.87E-05) | 40.81 (25.12) |
| Oxytetracycline hydrochloride | Antibiotic | 1 | 0.004 | 3.17E-03 | 7.11E-05 (1.06E-04) | 44.64 (29.92) |
| 1,2-Benzoisothiazolin-3-one | Microbicide; fungicide | 4 | 0.424 | 5.89E-02 | 7.78E-04 (2.00E-03) | 75.69 (29.48) |
| Didecyl dimethyl ammonium chloride | Biocide; disinfectant | 2 | 0.0139 | 4.13E-03 | 3.81E-05 (9.34E-05) | 108.34 (44.18) |
| Perfluorononanoic acid | Organofluorine | 1 | 0.601 | 2.39E-03 | 2.20E-05 (5.17E-05) | 108.39 (46.18) |
| Perfluorodecanoic acid | Organofluorine | 1 | 0.877 | 3.87E-03 | 3.46E-05 (4.66E-05) | 111.80 (82.95) |
| 4-(2-methylbutan-yl)phenol | Phenol | 1 | 0.634 | 2.31E-01 | 1.85E-03 (4.58E-03) | 125.23 (50.43) |
| Benzophenone | UV blocker; packaging | 1 | 0.306 | 4.85E-01 | 2.81E-03 (5.14E-03) | 172.37 (94.21) |
| Endrin | Organochlorine | 4 | 0.272 | 1.14E-03 | 6.55E-06 (9.97E-06) | 174.43 (114.51) |
| Gentian violet | Dye; topical antifungal drug | 1 | 0.01 | 9.99E-04 | 5.27E-06 (1.17E-05) | 189.56 (85.05) |

*aMHE, most highly exposed.  
*bAER, activity-to-exposure ratio.  
*cValues listed are associated with the most potent assay for each chemical. Values associated with other chemical-assay hits (where relevant) are listed in Supplementary Table S4.  
*dAll AERs returned for this chemical exceeded 100.
| Chemical | $C_{ss}$ (µM) | Assay Endpoint | $AC_{50}$ (µM) | Oral Equivalent (mg/kg/day) | $C_{ss} > 200$ (µM) | $AC_{50} < 0.5$ (µM) |
|----------|--------------|----------------|----------------|----------------------------|-------------------|-------------------|
| Heptadecafluorooctanesulfonic acid potassium salt | 217.01 | Inhibition of human CYP2C9 enzymatic activity | 1.30E-02 | 5.99E-05 | Yes | Yes |
| Mirex | 70.82 | Increased expression of prostaglandin E2 in human peripheral blood mononuclear cells | 1.14E-02 | 1.61E-04 | — | Yes |
| Diethylstilbestrol | 0.46 | Binding to human estrogen receptor | 7.43E-05 | 1.61E-04 | — | Yes |
| Diethylstilbestrol | 0.46 | Activation of estrogen receptor response element in transfected HepG2 cells | 1.01E-04 | 2.19E-04 | — | — |
| Diethylstilbestrol | 0.46 | Activation of estrogen receptor signaling pathway in transfected HEK293 cells | 1.27E-04 | 2.76E-04 | — | Yes |
| Potassium perfluorohexanesulfonate | 266.56 | Inhibition of human CYP2C9 enzymatic activity | 8.25E-02 | 3.09E-04 | Yes | Yes |
| Potassium perfluorohexanesulfonate | 266.56 | Inhibition of human CYP4F12 enzymatic activity | 8.60E-02 | 3.23E-04 | Yes | Yes |
| Diethylstilbestrol | 0.46 | Activation of estrogen receptor alpha signaling pathway in transfected HepG2 cells | 1.80E-04 | 3.92E-04 | — | Yes |
| Tannic acid | 0.34 | Inhibition of human GSK3b enzymatic activity | 2.00E-04 | 5.83E-04 | — | Yes |
| Dinoseb | 485.94 | Decreased mitochondrial membrane potential in HepG2 cells | 3.50E-01 | 7.20E-04 | Yes | — |
| Pentadecafluorooctanoic acid ammonium salt | 269.96 | Inhibition of human CYP2C9 enzymatic activity | 2.02E-01 | 7.48E-04 | Yes | Yes |
| Triphenyl phosphate | 0.79 | Binding to human peroxisome proliferator-activated receptor-gamma | 6.09E-04 | 7.66E-04 | — | Yes |
| Diethylstilbestrol | 0.46 | Activation of estrogen receptor signaling pathway in transfected HEK293 cells | 4.02E-04 | 8.73E-04 | — | Yes |
| Gentian violet | 10.01 | Decreased expression of interleukin-8 in human peripheral blood mononuclear cells | 1.00E-02 | 9.99E-04 | — | Yes |
| Gentian violet | 10.01 | Decreased expression of E-selectin adhesion protein in human endothelial cells | 1.00E-02 | 9.99E-04 | — | Yes |
| Gentian violet | 10.01 | Decrease expression of interleukin 1 alpha in human peripheral blood mononuclear cells | 1.00E-02 | 9.99E-04 | — | Yes |
| Endrin | 238.20 | Activation of estrogen receptor response element in transfected HepG2 cells | 2.72E-01 | 1.14E-03 | Yes | Yes |
| Dinoseb | 485.94 | Decreased expression of transforming growth factor-beta in human primary bronchial epithelial cells | 6.28E-01 | 1.29E-03 | Yes | — |
| 2-Methyl-4,6-dinitrophenol | 589.15 | Decrease mitochondrial membrane potential in HepG2 cells | 8.74E-01 | 1.48E-03 | Yes | — |
compared to the other chemicals. This suggests that the conser- 
vative assumptions employed in the IVIVE model limit our abil- 
ity to adequately predict blood $C_{ss}$ values for those chemicals 
that are highly cleared in vivo. Indeed, coumarin, flutamide, and 
lovastatin all possess in vivo blood $C_{ss}$ values of 0.01 mM or lower, 
down to 0.004 mM for flutamide. Of these 3 chemicals, the lowest 
predicted value was obtained for lovastatin, at 0.18 mM.

Additional work was performed to ascertain the impact of 
certain model assumptions and experimental design considera- 
tions on the predictive performance of the IVIVE. First, intesti- 
nal permeability data were obtained using the Caco-2 model 
and incorporated into the IVIVE to assess the impact of our 
assumption of 100% intestinal absorption. Caco-2 data 
assessed the predictive performance of 3 of the 16 chemicals 
assessed, although 2 of these 3 chemicals were already pre- 
dicted to be within 5-fold of the in vivo values using the conser- 
vative assumption. When these data are combined with 
equivalent data for Phase I chemicals (Wetmore et al., 2012), the 
assumption of 100% intestinal absorption appears to be 
adequate for over 85% of the chemicals, because incorporation 
of Caco-2 data significantly improved the predictions for only 4 
of the 29 chemicals assessed.

Use of pooled donor hepatocyte suspensions to measure 
hepatic clearance as performed here is considered to be the 
method of choice, as this system more accurately captures 
in vivo clearance than other available in vitro systems (Hallifax 
et al., 2010; Li et al., 1999; Pelkonen et al., 2013) while minimizing 
the impact of donor variability. However, hepatocyte suspen- 
sions are not suitable for quantitating clearance of low turnover 
compounds with $Cl_{in vitro} < 2 \mu l/(min \times 10^{6} cells)$, likely due to 
depletion of cofactor reserves over the 240 min time course 
(Houston et al., 2012). Three of the 16 chemicals for which no 
measurable clearance was detected were also assessed using 
plated hepatocytes over a 48-h time course. Clearance was 
detected in this more sensitive system and improved the IVIVE 
predictions, particularly for coumarin and diphenhydramine 
HCl (Table 1). However, use of plated systems requires consider- 
ation of additional factors. First, culture conditions are known 
to alter activity of cytochrome P450 enzymes, so attention to 
plating methods and characterization of enzyme activity should 
be monitored. Second, donor pools cannot be successfully used 
in these plated systems currently (Smith et al., 2012), so assess- 
ments across multiple donors need to be conducted to accu- 
rately determine variability in $Cl_{in vitro}$.

Inclusion of a range of pharmaceuticals and other chemical 
families (eg, organofluorines, persistent organic pollutants, etc) 
in the Phase II list provided an opportunity to assess the contribu- 
tion of potential bioactivities or chemical pharmacokinetics to 
relatively low OEDs relative with these compounds. Eleven chemicals 
(approximately 7% of total assessed) were identified as having an 
OED $< 1 \mu g/kg/day$, across 19 assay endpoints (Table 4). The main 
driver for a potent OED was $AC_{50}$ potency rather than a high $C_{ss}$.

Interestingly, only 2 of these 11 chemicals were drugs: the syn- 
thetic nonsteroidal estrogen diethylstilbestrol and Gentian 
blue, an antiseptic dye with antibacterial and antifungal proper- 
ties. Regardless, most of the assay hits were related to anti- 
-inflammatory and other drug target activity (eg, IL-8, IL-1α 
downregulation; CYP2C9, CYP4F12). The work described here 
uses presence and potency of a ToxCast hit—without regard for 
chemical mode of action or adverse outcome—as a conservative 
strategy that is appropriate in prioritization efforts. However, 
the context and nature of these activities will need to be more care- 
fully considered as related efforts—particularly those that go 
beyond prioritization—move forward.

The ToxCast and ExpoCast programs were designed to 
address the chemical safety needs of the USEPA through develop- 
ment and implementation of HT toxicity testing and expos- 
ure modeling strategies. By incorporating recent outputs of 
these 2 programs, this study provides an up to date assessment 
of the status of these efforts. It has also identified areas that 
warrant further attention. Refinement of HT hazard estimates 
to identify relevant modes of action and downstream adverse 
effects would arguably provide a more appropriate basis for a 
point of departure calculation than an approximation based on 
the most potent assay hit. Moreover, emergence of multiple HT 
probabilistic and traditional exposure modeling tools with a 
needed emphasis on near-field exposures (Isaacs et al., 2014; 
Wambaugh et al., 2013, 2014; Zhang et al., 2014) have under-
scoring the need for expansion and refinement of existing data 
sources that adequately capture chemical usage, product com- 
position, and functional information. With efforts already 
underway to address these limitations, this strategy is poised to 
undergo key refinements that will enable its utilization as part of a 
Tier 1 prioritization strategy (Thomas et al., 2013).

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SUPPLEMENTARY DATA

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