

## ABSTRACT

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### Incorporating Human Dosimetry and Exposure into the Utilization of ToxCast *In Vitro* Screening Data for Chemical Prioritization

"Under the direction of Drs. David J. Dix and Ivan Rusyn"

Humans are frequently exposed to chemicals that have undergone limited safety testing. To reduce the number of untested chemicals and prioritize limited testing resources, multiple governmental programs are using high throughput *in vitro* toxicity screens for assessing effects across multiple cellular pathways. In this study, metabolic clearance and plasma protein binding were experimentally measured for 39 of the 309 ToxCast Phase I chemicals. The experimental data was modeled using pharmacokinetics for estimating human oral equivalent doses that would produce steady state *in vivo* concentrations equivalent to ToxCast *in vitro* AC<sub>50</sub> values. The range of oral equivalent doses for the ToxCast assays was compared with human oral exposure estimates to assess whether *in vitro* bioactivity could occur at human exposure levels. Two of the 39 chemicals had overlapping oral equivalent doses and human exposures and would not have been identified using the rank potencies of the AC<sub>50</sub> values. These results demonstrate the importance of incorporating dosimetry and exposure when using high throughput *in vitro* data to identify the highest priorities for further testing and risk management.

## ACKNOWLEDGEMENTS

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## LIST OF ABBREVIATIONS

- AC<sub>50</sub> – Concentration at 50% activity
- ADME – Absorption, Distribution, Metabolism, Excretion
- AR - Androgen Receptor
- CAR – constitutive androstane receptor
- CDC – Centers for Disease Control
- cPAD – Chronic Population Adjusted Dose
- C<sub>ss</sub> – Steady state concentration
- CYP450 – cytochrome P450
- DMSO - dimethyl sulfoxide
- EDSP – Endocrine Disruptor Screening Program
- EPA – The United States Environmental Protection Agency
- ER – Estrogen Receptor
- EU – European Union
- FQPA – Food Quality Protection Act
- F<sub>ub</sub> – Plasma Unbound Fraction
- LOAEL – Lowest Observed Adverse Effect Level
- LogK<sub>hsa</sub> – QikProp log serum protein binding
- NCCT – EPA National Center for Computational Toxicology
- NCGC – NIH Chemical Genomics Center
- NET – Norepinephrine Transporter
- NHANES - The National Institutes of Health
- NIH- The National Institutes of Health

NOAEL – No Observed Adverse Effect Level

NTP – National Toxicology Program

OPP – Office of Pesticide Programs

PBPK – Physiologically based pharmacokinetic model

PK - Pharmacokinetic

POD – Point of departure

PXR – pregnane X receptor

REACH – The Registration, Evaluation, and Authorization of Chemicals

RED – Rapid Equilibrium Dialysis

RfD – Reference Dose

rTK – Reverse Toxicokinetic

SPE - Solid phase extraction

TR – Thyroid Hormone Receptor

TSCA - Toxic Substances Control Act

## Chapter 1

### Literature Review

#### *Chemical Landscape and Initiatives*

There are currently, around 10,000 environmental chemicals that have undergone limited toxicity testing (Judson et al., 2009a). The current testing regulations require *in vivo* toxicity testing that is time consuming, economically costly, and requires large numbers of animal lives. In an effort to reduce these costs, The United States Environmental Protection Agency (EPA) is undergoing a paradigm shift to prioritize these chemicals for targeted testing efforts. Advances in computational technology in combination with developments in molecular biology and chemistry have provided the ability to generate large data sets to study biological responses across dose ranges, more likely to be representative of human population exposures (Kavlock et al., 2008). For the purposes of evaluating these alternative testing methods, a concerted effort between the EPA, The National Institutes of Health (NIH) Chemical Genomics Center (NCGC), and the National Toxicology Program (NTP) are testing high-throughput research methods to generate *in vitro* data on thousands of environmental chemicals (Dix et al., 2007, Inglese et al., 2007, NTP 3004, Collins et al., 2008). Efforts are also being made by the European Union (EU) to increase the efficiency with which environmental chemicals undergo safety testing. Since 1981 only 2,700 new substances have entered the EU market, and approximately 80,000 chemicals are thought to be in use in the EU. Therefore, the large majority of these chemicals have limited or no toxicity information (Brown 2003).



### ***Regulatory Toxicology***

Regulatory toxicology saw its beginning in 1976 when the EPA generated the first phase of federal guidelines outlining approaches for assessing chemical safety. Risk assessments are based on a series of weight-of-evidence characterizations, dose response models, and exposure estimates (Anderson 1989). The EPA Office of Pesticide Programs (OPP) uses the National Research Council's four-step process for human health risk assessment. Step one of this process is hazard identification. Many different types of animal studies (examples include: reproductive, developmental, and chronic/cancer bioassays) are generally conducted to determine potential health outcomes that could result from chemical exposures. Step two involves an analysis of dose-response and is ultimately used to derive what is considered a safe value for humans (U.S. EPA, 2007). The dose response is used to derive a no observed adverse effect level (NOAEL). A NOAEL is an experimentally derived dose at which there was no statistically or biologically significant occurrence of an adverse health effect. A series of safety factors are applied as protective measures against inter- and intraspecies variability and the resulting value is referred to as the regulatory reference dose (RfD) (U.S. EPA, 1993). In 1996, the Food Quality Protection Act (FQPA) was signed into law and required an additional safety factor be applied to RfD values for protection of infants and children. The adjusted RfD is referred to as the chronic population adjusted dose (cPAD). Estimated levels of exposure that do not exceed 100% of the cPAD will not require mitigation efforts and are not of a level of concern for the agency (U.S. EPA, 2002).

### ***Pharmaco- and Toxicokinetics***

There are many challenges when drawing human *in vivo* relevance from *in vitro* activity measurements. In order to extend these approaches to quantitative risk assessments

and for prioritization efforts, it is necessary to understand the metabolic and pharmacokinetic potential of these chemicals (Judson et al., 2009b). Pharmacokinetic modeling has been used extensively in drug development and discovery. The implications of these models has led to fewer numbers of failed compounds and reduced the number of studies need for registration (Rajman 2008). The use of these models for drug development has provided an effective tool for capturing the inherent variability and uncertainty in the drug development process (Chien et al., 2005). Physiologically based pharmacokinetic models are gaining popularity; however, they require a certain amount of parameters that must be estimated (Slob et al., 1997). The use of *in vitro* estimates can help compensate for the lack of parameters presented in a single compartment model. Metabolic clearance assays measure the ability of a chemical to undergo biotransformation for the purposes of defining a pharmacological or toxicological profile for a chemical. These assays are frequently used in the pharmaceutical industry to investigate a key component of the absorption, distribution, metabolism, and excretion (ADME) properties and can aid in predictions of pharmacological outcomes (Masimirebwa 2003). A study conducted by McGinnity et al., tested 50 drugs to determine whether human hepatocytes measuring hepatic metabolic clearance would accurately predict *in vivo* human metabolic clearance. The results from this study demonstrate that intrinsic clearance measurements from cryopreserved human hepatocytes are statistically significantly correlated with fresh hepatocytes and *in vivo* human metabolic clearance (McGinnity et al., 2004). The degree to which a chemical is distributed through the body and made available for metabolism and excretion is due largely to its affinity for plasma protein. Studies have demonstrated that a strong correlation exists between the binding of a chemical and its lipophilicity (Lin et al., 1997). *In vitro* equilibrium dialysis has been shown to produce accurate estimates of unbound

fraction compared to plasma obtained from human subjects (Koike et al., 1985). The use of these methods to derive estimates of *in vivo* concentrations has not been greatly utilized for environmental chemicals.

### ***Estimating Human Exposures***

The incorporation of estimated human exposures should impact the order of chemical prioritization (Judson et al., 2009b). Biomonitoring data available from the U.S. Centers for Disease Control (CDC) and their National Health and Nutrition Examination Survey (NHANES) has become the largest dataset analyzing blood and urine concentrations over a wide range of subpopulations. Efforts have been made to extrapolate chemical concentrations in urine and blood to an oral intake value. This approach uses NHANES and other biomonitoring data and physiologically based pharmacokinetic (PBPK) models. The goals of this study were to help categorize chemicals into low, medium, and high priority for risk assessment purposes (Hays and Aylward, 2009). However, this approach does not estimate oral doses based on bioactivity from *in vitro* assays, and lacks the determination of health consequences from exposures. There are also limitations in using biomonitoring data to calculate exposures. Biomonitoring data only reflects the amount of the chemical in the body at the time of testing and may not represent original exposure levels. There also must be enough knowledge regarding tissue distribution and metabolic capacity in order to quantify exposure values (Juberg et al., 2008, NRC 2006). There have been efforts to use pharmacokinetic models to convert current regulatory RfD to biomonitoring equivalents. This could be used to evaluate how biomonitoring data compares to presumably safe levels as determined by the EPA. However, this approach is still basing human values off of rodent

toxicities. Overall, the current literature demonstrates the need for an additional comprehensive study using the methods described in this paper and the impact that these types of studies could have on regulatory toxicology.

## Chapter 2

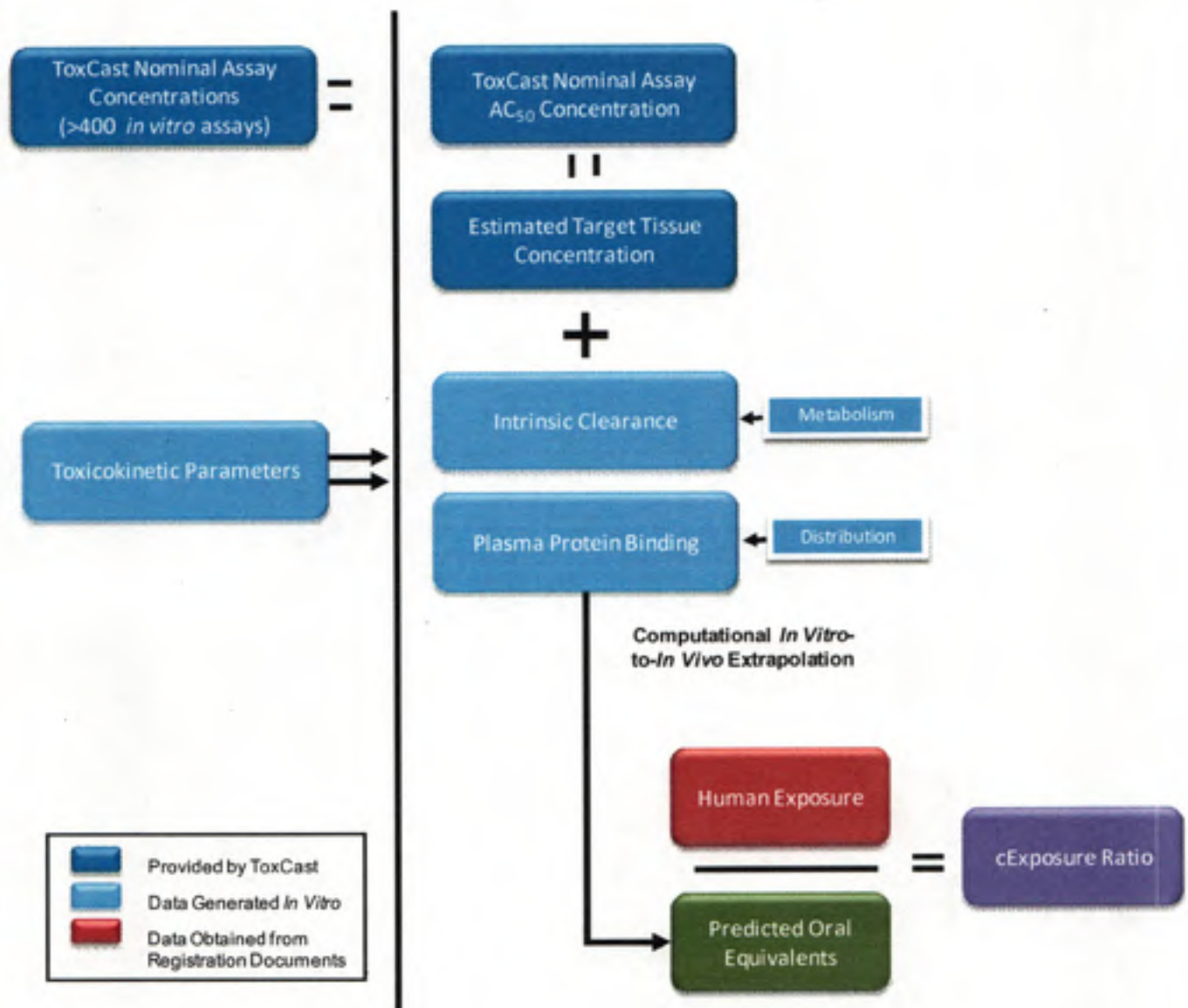
### Introduction

The current paradigm for testing agricultural and industrial chemicals for potential human health effects has not changed significantly over the past four decades. Tests typically include evaluation of carcinogenicity and chronic, developmental, and reproductive toxicities. The tests are inefficient, expensive, and rely heavily on experimental animals. Due to 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 (Judson *et al.* 2009a). This deficit in safety testing is beginning to be addressed through legislative mandates. In Europe, the Registration, Evaluation, and Authorization of Chemicals (REACH) initiative began a phased implementation in 2007 that will substantially alter the safety testing performed on both new and existing chemicals. In the United States, legislation to overhaul the Toxic Substances Control Act (TSCA) is currently under discussion in Congress. 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 (Hartung 2009; Breithaupt 2006; Hofer *et al.* 2004).

To address the large number of untested chemicals and improve chemical risk management, the U.S. EPA developed an alternative testing program called ToxCast™ to efficiently screen chemicals and prioritize limited testing resources toward those representing the greatest potential hazard to human health (Dix *et al.* 2007). In the first phase of the ToxCast program, hundreds of high-throughput *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.* 2009b). However, the use of only *in vitro* assay concentrations for prioritizing chemicals for testing may over- or under-estimate the potential hazard of these chemicals due to differences in bioavailability, clearance, and exposure *in vivo*.

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 was performed for a subset of ToxCast chemicals (Figure 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 then estimated the human oral equivalent doses that would be required to produce steady state *in vivo* concentrations equivalent to *in vitro* AC<sub>50</sub> values – that is, concentrations sufficient for 50% activity (see section “In Vitro Bioactivity Data” in Methods for details) – from the ToxCast data. For each chemical a human oral equivalent dose was estimated for each of the 398 ToxCast assays that produced a result. For human exposures, aggregate exposure estimates based on maximally allowed chemical residue levels from food and drinking water were obtained from registration documents. By comparing the human oral equivalent doses

across all the ToxCast assays with the exposure estimates, chemicals with the potential to perturb cellular pathways at relevant human exposure levels were identified.



**Figure 1. rTK Work Flow**

Schematic representation for incorporating human dosimetry and exposure into the high-throughput *in vitro* toxicity screening process performed in ToxCast.



## Chapter 3

### Methods

#### *Biochemicals*

HEPES buffer, gentamicin, penicillin/streptomycin, and Glutamax™ were purchased from Gibco (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 39 chemicals analyzed in this study represent a subset of the ToxCast Phase I chemical library (<http://www.epa.gov/ncct/toxcast/chemicals.html>). The subset was selected based on results from pilot studies measuring cellular cytotoxicity. As a whole, the ToxCast Phase I chemical library consists of 309 unique compounds selected based on a set of four criteria. First, compounds must have extensive chronic, cancer, multi-generational reproductive and developmental *in vivo* data available. This criterion was met by 95% of the ToxCast chemicals. Second, chemicals must be soluble in DMSO. This criterion was met by 97.5 % of the ToxCast chemicals. Third, chemicals must have a molecular weight between 250-1000 amu. This criterion was met by 90% of the ToxCast chemicals. Finally, chemicals must be obtainable with purity >90%. A total of 98.1% of the ToxCast chemicals met this criterion.

The 39 chemicals were obtained from various vendors, with 36 of the 39 chemicals possessing a purity exceeding 97%. Stock solutions (20 mM) were prepared in DMSO and stored in amber vials at -80°C. The stock solutions were diluted to the 0.2 and 2 mM working concentrations at the time the assays were conducted.

#### *Plasma Protein Binding Assay*

Human plasma was obtained from Bioreclamation, Inc. (Catalog No. HMPLEDTA2; Westbury, NY) and stored at -80°C until use. The plasma was isolated from six individual donors using K<sub>2</sub>EDTA as the anti-coagulant. The plasma was thawed at room temperature and centrifuged at 2000 X g for 10 min prior to analysis. Rapid equilibrium dialysis was conducted in 96-well format using the single-use rapid equilibrium dialysis plate (Catalog No. 90006, Pierce Biotechnology, Rockford, IL) according to manufacturer's instructions. Briefly, phosphate buffered saline (PBS) and plasma was 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 10 µM. The concentration of DMSO did not exceed 0.5%. The rapid equilibrium dialysis plate was wrapped in aluminum foil and incubated at 37°C at 100 rpm in an orbital shaker for 4 hours. 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 assays were performed in triplicate.

### *Metabolic Clearance Assay*

Cryopreserved hepatocytes, provided by CellzDirect/Life Technologies, Inc. (Durham, NC), were obtained from two separate pools of ten 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 quality control limits.

Incubation medium was prepared by supplementing William's E Medium with 0.1  $\mu$ M dexamethasone, 1 mL ITS+<sup>TM</sup> (per 500 mL medium), 4 mM Glutamax<sup>TM</sup>, 15 mM HEPES, and either 2  $\mu$ g/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., 2  $\mu$ M by adding 10  $\mu$ L 1 mM 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 non-coated polystyrene plate. The plates were then placed in an incubator at 37°C/5% CO<sub>2</sub> 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 X g for 6 minutes at 20°C. The cells were resuspended in incubation medium to a working cell density of  $1.0 \times 10^6$  viable cells per mL as determined by Trypan Blue<sup>TM</sup> exclusion. To each well of the incubation plates, 0.5 mL of  $1.0 \times 10^6$  viable cells per mL were added to yield a final cell density of  $0.5 \times 10^6$  viable cells per mL. A portion of the hepatocytes were 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  $\mu$ L aliquots were removed from each well and quenched with 50  $\mu$ L ice cold acetonitrile. Duplicate wells were run for each chemical. The quenched aliquots were frozen at  $-70^{\circ}\text{C}$  prior to analysis.

#### *Analysis by LC/MS*

Samples from the metabolic clearance assay were thawed at room temperature, vortexed briefly and centrifuged at 6,000 X g for 1 min. Samples were then diluted 1:10 with 5% v/v acetonitrile in water, mixed, and centrifuged for 1 min at 6,000 X g. Samples were analyzed on an API 3000 LC/MS (Applied Biosystems, Foster City, CA).

Samples from the plasma protein binding assay were thawed at room temperature, vortexed briefly, and centrifuged at 10,000 X g for 10 min. Samples were diluted in 5% v/v acetonitrile in water, mixed, and centrifuged at 6,000 X g for 1 min prior to analysis on the API 3000 LC/MS.

#### *Analysis by GC/MS*

All samples were thawed at room temperature, vortexed briefly, and centrifuged at 10,000 X g for 10 min. Solid phase extraction (SPE) was conducted using Sep-Pak C<sub>18</sub> 96-well SPEplates (Cat. # 186003966; Waters, Milford, MA) in conjunction with a vacuum manifold following manufacturer's instructions. Samples were eluted in methanol into 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.

#### ***Plasma Protein Binding Data Analysis***

To calculate percent of unbound chemical ( $F_{ub}$ ), the test compound concentration in the PBS chamber was divided by the concentration in the matched plasma sample and multiplied by 100. For a subset of compounds, the calculations resulted in either >100% or 0%  $F_{ub}$ . To estimate the  $F_{ub}$  for these chemicals, the Qikprop software application (Version 3.0, Schrödinger, New York, NY) was used to predict the log serum protein binding ( $K_{hsa}$ ). Statistical analysis of the significance of the correlation coefficient was performed using the standard test for correlation with  $t$  distribution and  $n-2$  degrees of freedom.

#### ***Metabolic Clearance Data Analysis***

The metabolic clearance results for the 1 and 10  $\mu$ M starting concentrations were plotted separately in semi-log format (log concentration versus time) with two replicates at each time point. The time course data at each concentration was 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  $\mu$ l/min/ $10^6$  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 hour time point, the degrees of freedom were adjusted accordingly. For chemicals that fell below detection levels before the 2 hour 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.

### ***In Vitro Bioactivity Data***

The ToxCast program measured activity of 309 compounds against a set of ~500 *in vitro* assays using 9 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 dose response format and from each dose-response curve, it was determined whether the chemical was active or not, and if it was, a characteristic concentration was determined. These were with  $AC_{50}$  values (the concentration at which 50% of maximum activity was seen) or LEC values (the lowest effective concentration at which activity was statistically significantly above that seen in negative controls). The data is described in Judson et al 2009a, and all data is available from the ToxCast web site (<http://www.epa.gov/ncct/toxcast>).

### ***Estimation of Oral Equivalentents using In Vitro-to-In Vivo Extrapolation***

Simulations were performed for each chemical and each  $AC_{50}$  / LEC value across 398 *in vitro* assays – inactive chemical-assay combinations were not simulated. The *in vitro* bioactivity was assumed to be solely the result of the parent compound. For each compound, an empirical one-compartment pharmacokinetic model was parameterized. There were two routes of elimination: metabolism (determined from measured metabolic clearance) and renal excretion (estimated by multiplying the measured  $F_{ab}$  by the normal adult glomerular filtration rate ( $111 \text{ mL/min}/1.73 \text{ m}^2 = 6.7 \text{ L/hr}$ ) (Rule *et al.* 2004)). Gastrointestinal absorption was assumed to be 100%.

Metabolic clearance and plasma protein binding measurements were entered into the Simcyp software application (Simcyp Limited, Sheffield, UK) (Jamei *et al.* 2009). For the metabolic clearance values, either the 1 or 10  $\mu\text{M}$  value was used depending on which value was closer to the  $\text{AC}_{50}$  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 10  $\mu\text{M}$  and used as input. If the experimentally measured  $F_{ub}$  was  $>100\%$  or  $0\%$ , the *in silico* QikProp predicted  $F_{ub}$  was used.

Simulations were performed using a dose of 1 mg/kg/day. Monte Carlo analysis was performed within the Simcyp software to simulate variability across a population of 100 healthy individuals of both sexes from 20-50 years of age. A coefficient of variation of 30% was used for intrinsic and renal clearance. The median, upper and lower 5<sup>th</sup> percentile 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:

$$\text{Oral Equivalent (mg/kg/day)} = \text{ToxCast AC50 } (\mu\text{M}) \times \frac{1 \text{ mg/kg/day}}{C_{ss} (\mu\text{M})}$$

### *Pharmacokinetic Modeling*

Published pharmacokinetic (PK) data for triclosan, 2,4-dichlorophenoxyacetic acid, oxytetracycline dihydrate, and bisphenol A, and a PBPK model for parathion were used to estimate the  $C_{ss}$  in the plasma in humans exposed to 1 mg/kg/day (SCCP 2009, Gentry *et al.* 2002; Kohli *et al.* 1974; Sauerhoff *et al.* 1977; Volkel *et al.* 2002)

### *Estimation of Human 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 decision 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 supplemental material (Supplemental Table 5). Estimated exposure for Bisphenol A was calculated using NHANES urinary concentration of total Bisphenol A. Concentrations were then back-calculated by multiplying by 24 hour urinary output volume (NTP-CERHR, 2008). The highest exposed subgroup, ages 12-19, was used in the in the data analysis.

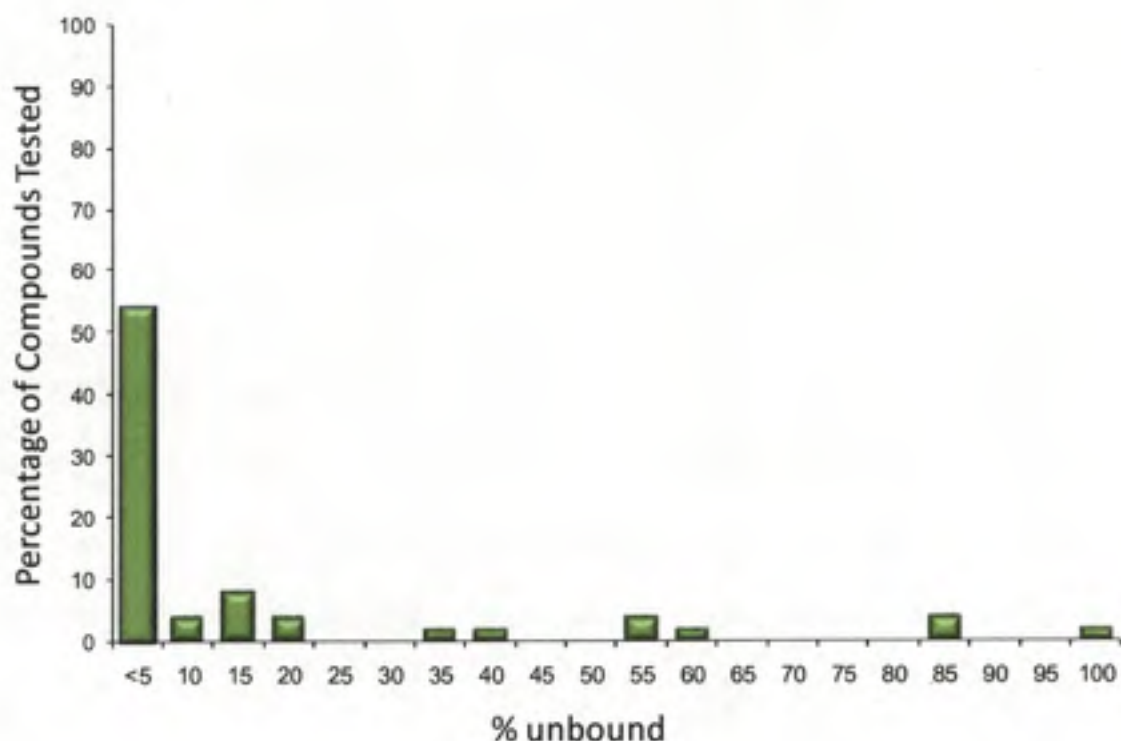


## Chapter 4

### Results

#### *Plasma Protein Binding*

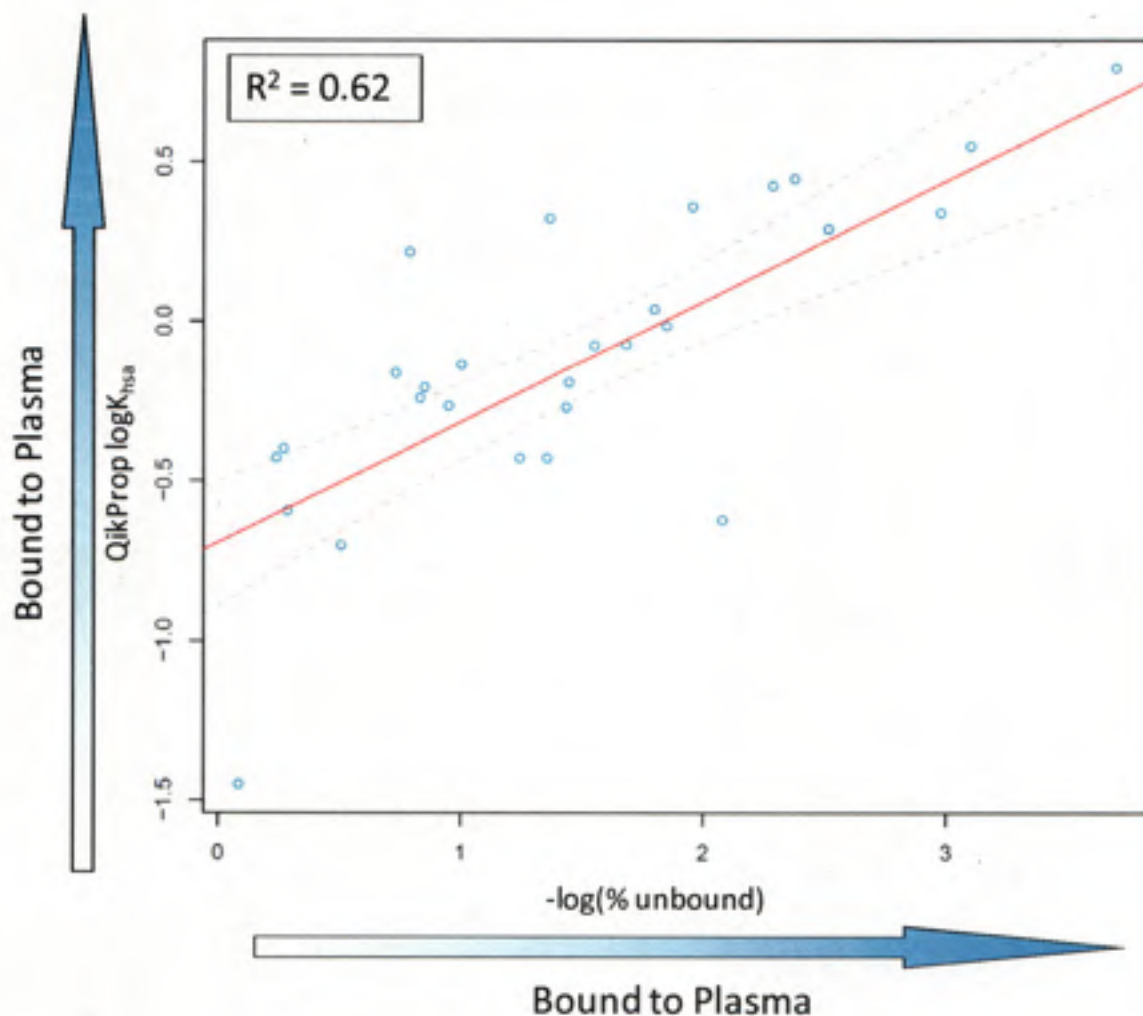
Measurements of plasma protein binding were performed at 1  $\mu\text{M}$  and 10  $\mu\text{M}$  for each chemical. Assuming that binding to plasma proteins is unlikely to be saturated at the concentrations tested and the minimal differences observed between the  $F_{ub}$  values at 1 and 10  $\mu\text{M}$ , 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 (Figure 2). Four chemicals (Dibutyl phthalate, Vinclozolin, Monobutyl Phthalate, and Nitrapyrin) were not detected by gas chromatography-mass spectrometry. For four chemicals (Etoxazole, MGK, Parathion, and Triclosan) there was no unbound chemical detected at either the 1  $\mu\text{M}$  or 10  $\mu\text{M}$  concentration. It is possible that these chemicals were bound to the membrane present in the dialysis device thereby reducing the amount of unbound chemical. In addition, two chemicals, Dichlorvos and Chlorpyrifos-oxon, produced  $F_{ub}$  results of greater than 100%. These results were likely due to the breakdown of the chemical by esterases present in the plasma samples.



**Figure 2. Distribution of Plasma Protein Binding**

Distribution of plasma protein binding measurements across both the 1 uM and 10 uM concentrations for the 39 ToxCast chemicals analyzed.

To assess the possibility of using *in silico* predictions of plasma protein binding for the 11 chemicals where experimental measurements failed, the QikProp software application was used to predict binding to human serum albumin for the 28 chemicals where experimental measurements of binding were obtained. The results showed that predictions generally matched experimental measurements with a correlation coefficient ( $r^2$ ) of 0.62 ( $p < 0.0001$ )(Figure 3). Based on these results, the QikProp *in silico* predictions were used for the 11 chemicals. Both chemicals suspected of interactions with esterases ( $>100\%$   $F_{ub}$ ) were predicted to bind to plasma *in silico* with  $>80\%$   $F_{ub}$ . The four chemicals that demonstrated experimental values of  $0\%$   $F_{ub}$  all had *in silico* values of  $< .57\%$   $F_{ub}$ , providing additional support for the experimental values.



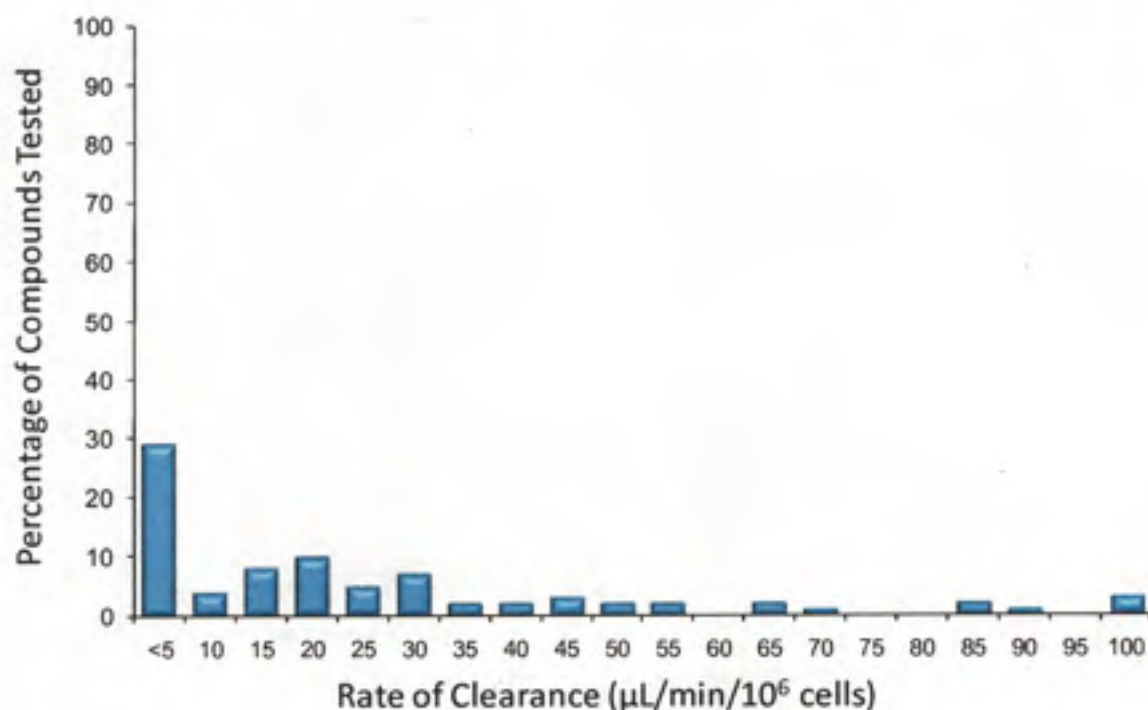
**Figure 3. Correlation between *in silico* and *in vitro* Plasma Protein Binding**

Scatter plot and linear regression analysis of the *in silico* predicted binding to human albumin ( $-\log K_{hsa}$ ) and the experimentally measured plasma protein binding ( $-\log F_{ub}$ ). The *in silico* predictions were obtained with Qikprop.

#### ***Metabolic Clearance***

The results from the metabolic clearance measurements ranged from 250.3 to -12.3  $\mu\text{l}/\text{min}/10^6$  cells. The negative values reflect metabolic saturation and variability in the experimental measurements. Of the 12 chemicals that showed metabolic saturation at the 10

$\mu\text{M}$  concentration, only four (acetamiprid, bromacil, emamectin, and bentazone) were also saturated at the 1  $\mu\text{M}$  concentration. The distribution of metabolic clearance measurements was similar to the plasma protein binding measurements (Figure 4); however, analysis demonstrated no significant correlation between metabolic clearance and  $F_{ub}$  ( $r^2 = 0.007$ ).



**Figure 3. Distribution of Metabolic Clearance**

Distribution of metabolic clearance measurements across both the 1  $\mu\text{M}$  and 10  $\mu\text{M}$  concentrations for the 39 ToxCast chemicals analyzed.

#### *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 and 2,4-dichlorophenoxyacetic acid, the *in vitro* predicted  $C_{ss}$  value was within the range of the human kinetic studies. For oxytetracycline-dihydrate, the *in vitro* model over-predicted the  $C_{ss}$  value calculated from human PK data. One explanation for this

over-prediction is the assumption of 100% oral absorption in the *in vitro* models, since oxytetracycline-dihydrate's, oral bioavailability has been reported to be less than 10% (Bjorklund and Bylund 1991; Nielsen and Gyrd-Hansen 1996). For parathion, the *in vitro* model prediction is close to the  $C_{ss}$  estimated with the PBPK model.

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, while the experimental data was 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.

**Table 1 Distributions of Oral Equivalent Values and Predicted Chronic Exposures**

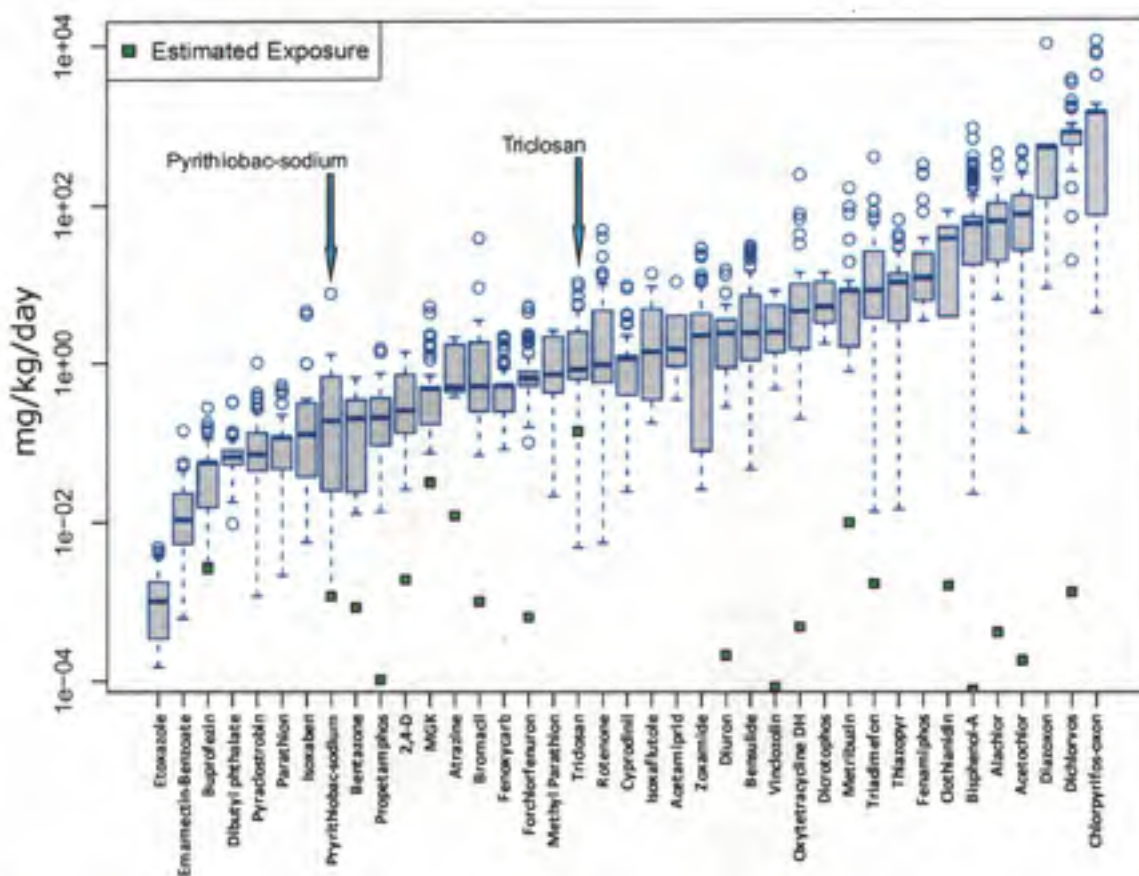
Chemical	PK- or PBPK-Derived $C_{ss}$ <sup>a</sup> ( $\mu$ M)	<i>In Vitro-to-In Vivo</i> Extrapolation $C_{ss}$ <sup>a</sup> ( $\mu$ M)
2,4-Dichlorophenoxyacetic acid	9.05-90.05	40.77
Oxytetracycline-dihydrate	0.36	2.02 <sup>b</sup>
Triclosan	2-10	3.68
Bisphenol A	<0.13	0.42
Parathion	0.17	0.15

<sup>a</sup> $C_{ss}$ , concentration at steady state

<sup>b</sup> Does not consider low oral bioavailability

Using the *in vitro-to-in vivo* modeling approach, oral equivalent doses were determined for each of the 39 chemicals across all of the 389 ToxCast assays that possessed a measurable  $AC_{50}$  value. For example, among the 39 chemicals, Emamectin benzoate had  $AC_{50}$  values for 127 assays while Acetamiprid had  $AC_{50}$  values for 6 assays. In the modeling simulations, Monte Carlo analysis was performed to simulate variability in the  $C_{ss}$  across a cohort of 100 healthy

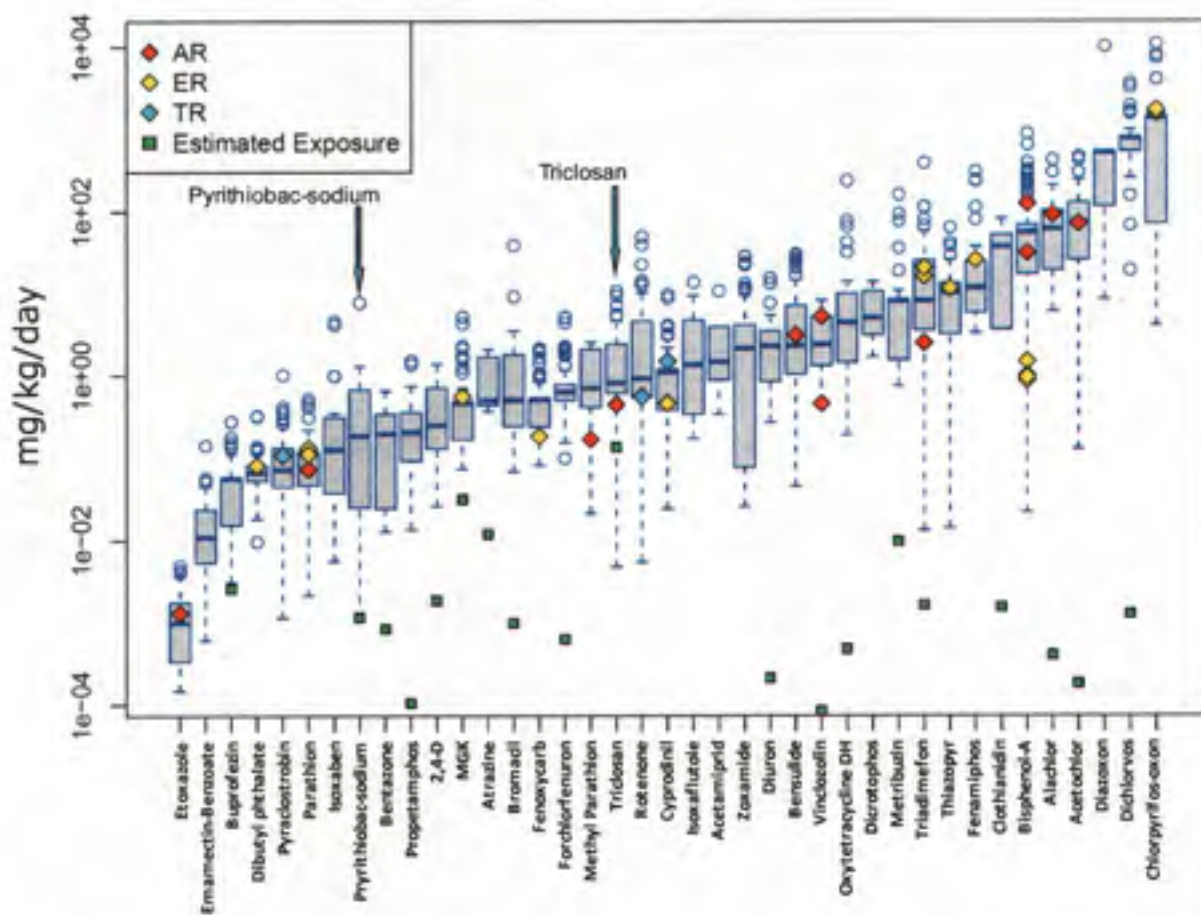
individuals of both sexes from 20-50 years of age. From the Monte Carlo analysis, the upper 95<sup>th</sup> percentile of the  $C_{ss}$  was used to obtain the most conservative estimate of the oral equivalent doses for a population. The oral equivalent doses were then summarized as box plots representing the range of values over all the ToxCast assays (Figure 5). From the chemicals tested, Etoxazole had the lowest oral equivalent doses with a median of 0.006 mg/kg/day and a minimum of 0.00022 mg/kg/day. The chemical that generated the highest oral equivalent doses was Chlorpyrifos-oxon with a median oral equivalent of 1361.3 mg/kg/day.



**Figure 5. Distributions of Oral Equivalent Values and Predicted Chronic Exposures** Comparison of oral equivalent doses and estimated human exposure levels for the 39 ToxCast chemicals analyzed. The oral equivalent doses (mg/kg/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 20-50 years of age. From the Monte Carlo simulations, the lower 5<sup>th</sup> 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 limits and the entire range. The human exposure estimates for 24 chemicals were obtained from registration documents and reflect the most highly exposed group or subpopulation (green squares). Exposures for dicrotophos, fenamiphos, and methyl parathion were below  $1e^{-5}$  mg/kg/day and are therefore not shown on the graph. Chemicals where the human exposure values fall within the range of predicted oral equivalents are highlighted with arrows.

Individual assays can also be assessed in order to prioritize for specific targeted testing efforts. For example, the estrogen receptor-alpha (ER), androgen receptor (AR), and thyroid hormone receptor (TR) have been identified by the EPA Endocrine Disruptor Screening Program (EDSP) as being key targets in identifying potential candidates for endocrine disruption. Figure 6 shows the relationship of estimated human exposure to oral equivalent values representing perturbations of these three molecular targets. The ratio of distances from the target activity/activities and exposure can be used to rank order chemicals.



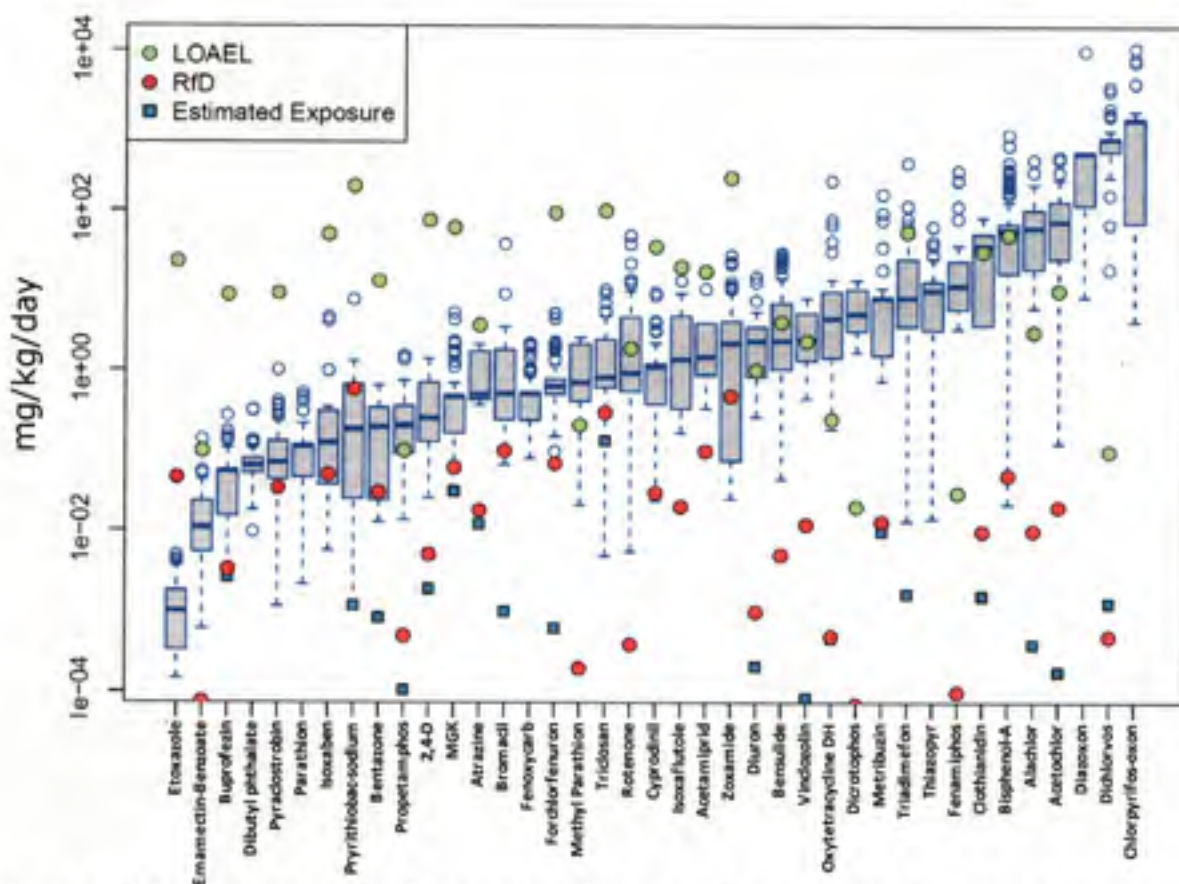
**Figure 6. Distributions of Oral Equivalent Values and Endocrine Relevant Bioactivity**  
 Comparison of oral equivalent doses of ToxCast assays identified as relevant for prioritizing for endocrine disruption are presented here. The ToxCast assays identified include 4 androgen receptor (AR) assays: NVS\_NR\_hAR, NCGC\_AR\_Antagonist, NCGC\_AR\_Agonist, NVS\_NR\_rAR; 5 estrogen receptor (ER) assays: ATG\_ERE\_CIS, NCGC\_ERalpha\_Agonist, ATG\_ERa\_TRANS, NVS\_NR\_bER, NVS\_NR\_hER; and one thyroid hormone receptor (TR) assay: ATG\_THRa1\_TRANS. The human exposure estimates for 24 chemicals were obtained from registration documents and reflect the most highly exposed group or subpopulation (green squares).

None of the estimated human exposures overlapped with oral equivalents for ER, AR, or TR.

Some chemicals were not active in any of these assays, and therefore, do not have any endocrine representation. Triclosan had the narrowest margin of estimated exposure (0.13 mg/kg/day) and endocrine activity (0.44 mg/kg/day) for the NVS\_NR\_hAR assay.



An additional analysis was conducted plotting rodent lowest observed adverse effect levels (LOAEL) and RfD values tabulated in each reregistration eligibility decision document (Figure 7).



**Figure 7. Distributions of Oral Equivalent Values and Regulatory Decisions Based on Chronic Rodent Studies**

Comparison of oral equivalent doses of ToxCast assays are plotted with rodent LOAEL (green circles) and chronic RfD (red circles) values. The human exposure estimates for 24 chemicals were obtained from registration documents and reflect the most highly exposed group or subpopulation (blue squares).

The majority of chemicals did not have LOAEL values that overlapped with estimates of *in vivo* bioactivity. 12 chemicals produced LOAEL values in rodent studies that were within the distributions of estimated human *in vivo* oral equivalents and 4 chemicals have regulatory LOAEL values below any estimate of human bioactivity measured in the ToxCast assays.

For 11 of 39 chemicals, RfD values were within oral equivalent values from ToxCast data. etoxazole was the only chemical with an RfD greater than all generated oral equivalent values.

### *Human Exposures*

Aggregate human exposure estimates based on maximally allowed chemical residue levels from food and drinking water were obtained from registration documents for 24 of the 39 chemicals (Appendix A). For most chemicals, the estimated aggregate 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, pyriithiobac-sodium and triclosan, showed overlap in the values (Figure 5). 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 pyriithiobac-sodium at levels below estimated exposures. The estimated exposure for triclosan is 0.13 mg/kg/day. Out of a total of 86 ToxCast assays which produced AC50s for triclosan, the CLZD\_CYP2B6\_24, ACEA\_LOCdec, and NVS\_TR\_hNET assays overlapped with the estimated exposure due to oral equivalents of .0048, .0065, and .043 mg/kg/day, respectively. Therefore, exposure levels are estimated to be occurring at 28, 21, and 3.1 fold greater than predicted bioactivity, respectively. The estimated exposure for pyriithiobac-sodium is .0012 mg/kg/day. A total of 15 ToxCast assays produced AC50s for pyriithiobac-sodium, with overlap for the CLZD\_SLCO1B1\_48 assay due to an oral equivalent of .0011 mg/kg/day. Therefore, the exposure level is estimated to be essentially equal with the oral equivalent (Table 2).

**Table 2.** *In vitro* assays with oral equivalent doses below that of the estimate human exposure

Chemical	Assay	Endpoint	AC <sub>50</sub> ( $\mu$ M)	Oral Equivalent Dose (mg/kg/day) <sup>a</sup>	Human Exposure (mg/kg/day) <sup>b</sup>
Triclosan	CLZD_CYP2B6_24	CYP2B6 mRNA in Primary Human Hepatocytes (24 h)	0.034	0.0048	0.13
Triclosan	ACEA_LOCdec	Cellular impedance measuring alterations in cell morphology and cell survival	0.046	0.0065	0.13
Triclosan	NVS_TR_hNET	Competitive binding of the human norepinephrine transporter	0.31	0.043	0.13
Pyriithiobac- sodium	CLZD_SLC01B1_48	SLCO1B1 mRNA in Primary Human Hepatocytes (48 h)	0.067	0.0011	0.0012

<sup>a</sup>Oral equivalent dose for the lower 5th percentile of a cohort of 100 healthy individuals of both sexes from 20-50 years of age.

<sup>b</sup>Aggregate human exposure from food and drinking water sources for the most highly exposed group or subpopulation.

## Chapter 5

### Discussion, Limitations, Conclusions and Future Directions

#### *Discussion*

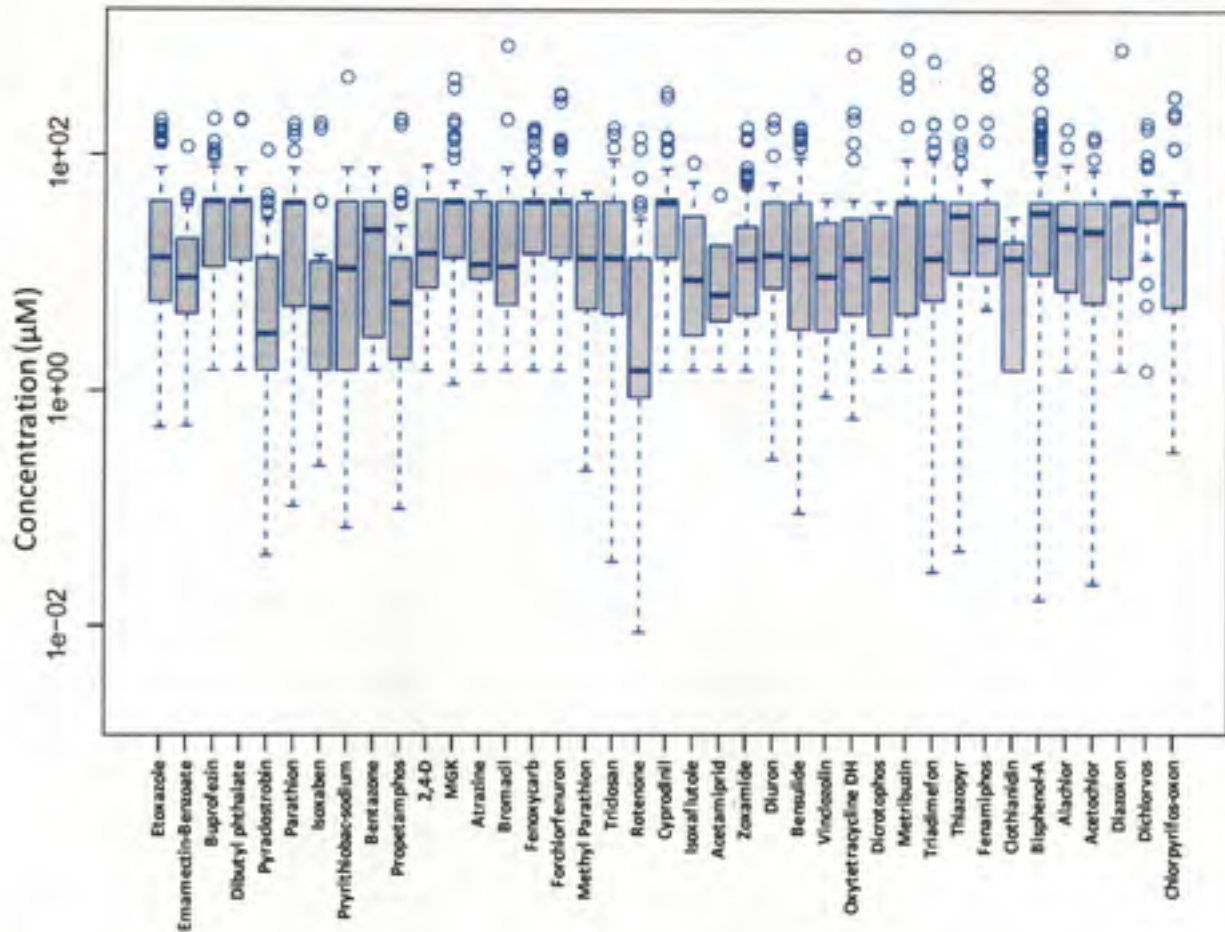
Over the past five years there has been a far reaching discussion on the future direction of toxicology and how chemical testing is performed (Collins *et al.* 2008; Dix *et al.* 2007; NRC 2007). One suggestion has focused on the use of high throughput *in vitro* screening to assess responses across multiple assays and prioritize compounds for conventional *in vivo* testing. Current efforts are underway in both the United States and Europe to assess the utility of high throughput *in vitro* methods to efficiently screen chemicals and prioritize limited testing resources (e.g., ToxCast and Tox21 (Kavlock *et al.*, 2009)). In these high-throughput screening activities, most of the initial effort has been focused on characterizing the biological activity of agricultural and industrial chemicals across multiple cellular pathways and processes. Less attention has been paid to determining the relationship between concentrations of the chemical active *in vitro* and expected concentrations in human populations. Pharmacokinetic properties and human exposure characteristics are equally important as the biological activity in determining a chemical's risk to human health.

In this study, *in vitro* assays were performed on a subset of ToxCast chemicals to estimate two critical determinants of pharmacokinetics, 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 to other methods, such as microsomal incubation (Jones and Houston 2004). For the chemicals evaluated in this study, 29 of 39 showed significant metabolic clearance in at least one of the two concentrations tested. Plasma protein binding was examined using equilibrium dialysis, which has become a standard method for measuring binding in high-throughput studies (Waters *et al.* 2008). Greater than 50% of the chemicals were highly bound to plasma proteins. Based on this sample of chemicals, the results suggest that while most ToxCast chemicals are metabolically labile, binding to plasma proteins may limit the availability for metabolism or renal clearance and would increase the effective half life. An exception to this generalization would be the case of chemicals that are subject to active renal resorption, which would lead to a higher steady-state plasma concentration at a given dose.

The metabolic clearance and plasma protein binding measurements were used together with *in vitro*-to-*in vivo* extrapolation modeling to predict the pharmacokinetic 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 pharmacokinetics of candidate molecules. 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 39 chemicals across all of the 389 ToxCast assays that possessed a measurable  $AC_{50}$  value. The range of oral equivalent doses for each chemical represent the amount a person in the lowest 5th percentile would have to

consume The rank order of the chemicals based on the oral equivalent doses was significantly different from the order based on the  $AC_{50}$  values (Figure 8), which suggests that prioritization based solely on  $AC_{50}$  values can misrepresent the potential hazard of chemicals.



**Figure 8. Distributions of  $AC_{50}$  activity across all ToxCast assays by Chemical**

Box plots of  $AC_{50}$  values across the 398 ToxCast assays for the 39 ToxCast chemicals analyzed. The chemicals were sorted based on order of chemicals in Figure 5.

Aggregate human exposure estimates were obtained for each chemical 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 human exposures across the 39 chemicals,

the human exposures were well below the range of estimated oral equivalent doses for most chemicals. This study used the AC50 as a measure of significant biological activity. There is no universal method to relate *in vitro* potency to *in vivo* activity; hence other values, e.g. AC20 or AC75, could be calculated and used in this approach. For the purposes of demonstrating the application of the approach to chemical prioritization, the current method utilized the AC50. With this value, there was a margin between exposure and effective dose for almost all chemicals, even though comparisons were made between the most highly potentially exposed human subpopulation and oral equivalent doses for the lowest 5<sup>th</sup> percentile of a simulated human cohort. Only two chemicals, pyriithiobac-sodium and triclosan, were identified where the oral equivalent doses overlapped estimated human exposures. These two chemicals would not have been identified from the *in vitro* AC<sub>50</sub> values, the oral equivalent doses, or the exposure estimates alone. In general, it will be necessary to have information on hazard and *in vitro* dose-response coupled with exposure information to develop testing priorities. The linkage of hazard identification, dose-response, and exposure are the cornerstones of risk assessment (NRC, 1983). Triclosan is a broad spectrum antibacterial and antifungal agent that is present in many household items with a high potential for human exposure (e.g., soap, toothpaste). The estimated highest human exposure for triclosan (0.13 mg/kg/day) is in excess of the oral equivalent values for three ToxCast assays – ACEA\_LOCdec (0.0065 mg/kg/day), CLZD\_CYP2B6\_24 (0.0048 mg/kg/day), and NVS\_TR\_hNET (0.043 mg/kg/day). The ACEA\_LOCdec assay is a measure of decreased cell viability. The CLZD\_CYP2B6\_24 assay indicates expression of CYP2B6 in primary human hepatocytes (Rotroff *et al.* 2010). The expression of CYP2B6 is regulated by the constitutive androstane receptor (CAR) and pregnane X receptor (PXR), and

studies have identified triclosan as a human PXR activator (Jacobs et al. 2005). At high doses, triclosan disrupts rat thyroid hormone homeostasis *in vivo* (Crofton et al. 2007, Zorrilla et al. 2009) and recent work suggests CAR/PXR activation may play a role in this disruption (Paul et al. 2010). The NVS\_TR\_hNET assay is a competitive binding assay for the human norepinephrine transporter (NET). Although not well understood, there appears to be some relationship between thyroid hormones and NET – a recent study found that thyronamines act as norepinephrine uptake inhibitors, causing an increase in the amount of norepinephrine that can be found at the synaptic cleft (Snead et al., 2007). The relationship of these *in vitro* bioactivities to any potential modes of action leading to adverse effects remain unclear, and this will be one of the ongoing challenges to interpreting *in vitro* bioactivity relative to potential hazard and risk.

Pyriithiobac-sodium is an herbicide that is used in the cotton industry. Within standard registration studies, pyriithiobac-sodium had no evidence of genotoxicity, and was not teratogenic in rats or rabbits; however, a 23-month rat chronic/carcinogenicity study demonstrated toxic effects in males including focal cystic degeneration in the liver and kidney effects in females at 200 and 918 mg/kg/day, respectively. Pyriithiobac-sodium is listed as a Group C possible human carcinogen due to the presence of adenomas, carcinomas and combined adenoma/carcinomas in the male mouse liver and rat kidney (Federal Register, 2002). Among the ToxCast assays, pyriithiobac-sodium increased expression of SLCO1B1 in primary human hepatocytes with an oral equivalent dose (0.0011 mg/kg/day) approximately equal to the highest estimated human exposure (0.0012 mg/kg/day). SLC01B1 is the gene that encodes for solute carrier organic anion transporter family member 1B1 (OATP1B1), which is responsible for hepatic uptake of a variety of compounds (Pasanen et al., 2008).



Again, the relationship of these bioactivities in regard to hazard and risk is unclear and will require additional research.

The oral equivalent values of ToxCast assays measuring bioactivity of three molecular targets (ER, AR, and TR) identified by the EPA EDSP program as being relevant for assessing endocrine toxicity *in vitro* were plotted in Figure 6. There were no estimated exposure levels that overlapped with any of these endocrine assays. Triclosan had the narrowest margin, 0.15 mg/kg/day, of estimated human exposure for the NVS\_NR\_hAR. This assay measures competitive binding of the human androgen receptor in an immortalized cell line. Through the identification of specific assays that represent alteration of target pathways, in this case endocrine pathways, can aid in the prioritization of chemicals for targeted testing. Regulatory reproductive toxicity testing is among the most expensive in the terms of monetary costs and animal lives (Novic and Vracko, 2010). The approach described by Figure 6 identifies a method capable of identifying high priority chemicals for reproductive or developmental toxicity testing and could reduce the number of chemicals that undergo unnecessary testing.

The current regulatory process uses the doses resulting in frank toxicity from rodent bioassays to ultimately arrive at levels considered to be safe for humans. Figure 7 shows the same oral equivalent box plots representing human bioactivity compared to overt toxicity observed in rodent studies. Intuitively, one would expect to observe molecular perturbations at dose levels prior to those manifesting into an adverse phenotypic outcome. This dynamic is represented across the majority of chemicals; however, 11 chemicals have LOAEL values below the median oral equivalent. A possible explanation for this is that species differences exist resulting in adverse effects in rodents that may not be relevant for humans. RfD values

are derived from the NOAEL and are considered to be the maximum acceptable oral dose for a chemical substance. These values are also plotted in Figure 7 and the majority of these RfD are set at levels below oral equivalent values. However, 11 RfD values overlapped with *in vivo* estimates of bioactivity, suggesting that using only animal bioassays may over- or underestimate what levels of exposure would result in adverse human effects.

### ***Limitations***

The pharmacokinetic approaches presented in this study have the potential to extend the hazard identification paradigm towards the use of *in vitro* data in a risk assessment context. Nonetheless, there are several 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 are largely conservative from a human health standpoint and would result in a lower estimate of the oral equivalent dose required to achieve a specific steady state plasma concentration. Second, the use of AC<sub>50</sub> values as the basis for calculating the oral equivalent doses may not represent the minimum concentration of the chemical required to observe a biological effect. The AC<sub>50</sub> value has become the standard metric for comparing potencies of chemicals, but may not be appropriate for identifying biologically relevant concentrations for prioritization and risk assessment. More conservative estimates may be derived from statistical methods that assess significant deviations from the vehicle treated controls. Third, chemicals may preferentially partition

into a specific tissue, potentially resulting in sequestered concentrations greater than would be determined for the blood alone. Fourth, the *in vitro* assays may contain some of the pharmacokinetic features being determined with the metabolic clearance and protein-binding assays, i.e. cellular assays may have significant biotransformation capacity and medium may have high serum protein concentrations. Hence, these pharmacokinetic parameter may already be reflected in the AC<sub>50</sub> determined by a particular *in vitro* assay and application of the additional metabolism and protein-binding measurements may over-compensate for these effects. Fifth, the *in vitro* assay activity likely reflects activity of the parent molecule; activation to an active metabolite occurring *in vivo* may not be detected with this approach. Finally, the use of highly sensitive *in vitro* assays for predicting relevant responses in humans is debatable. Activation of these *in vitro* endpoints does not necessarily represent an adverse biological response, but should be regarded as a measure of putative 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

### ***Conclusions***

The results of this study have significant 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<sub>50</sub> 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<sub>50</sub> values for prioritization could under- or overestimate the risks associated with other chemicals because of inadequate dosimetry estimates due to 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 is critical for informed decisions on chemical testing priorities and regulatory attention. 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).

### ***Future Directions***

This experiment is currently being run on the rest of the ToxCast phase I chemicals. This experiment will provide a complete dataset of valuable information on metabolic clearance and plasma protein binding. This data will also be incorporated into a prioritization effort in conjunction with additional toxicological pathway information. 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 an adverse response and an oral equivalent dose could be estimated. This would provide the ability to derive a point of departure (POD) that could parallel the current risk assessment methods. This data will also be used to determine whether adjusting AC<sub>50</sub> values for pharmacokinetic parameters can improve the predictive power of the *in vitro* assays when compared to rodent legacy data.

With the proper validation this could enhance current risk assessments by incorporating data from human cells as opposed to extrapolations made from rodent toxicity

tests. These methods would also be derived from molecular bioactivity instead of coarse grained *in vivo* tests.

One of the major challenges in developing these predictive signatures is the issue of species extrapolation from human *in vitro* assays to rodent *in vivo* data. In an additional collaboration between The EPA National Center for Computational Toxicology (NCCT) and the Hamner Institutes for Health Sciences, this experiment will be repeated using rodent plasma protein and cryopreserved hepatocytes. The pharmacokinetic model will be adjusted to represent the rat physiology. This experiment could greatly improve predictive signatures because it will allow for direct comparison for rodent pharmacokinetics and bioactivity with historical *in vivo* toxicity testing endpoints.

## Chapter 6

### Practicum Report

The first phase of my practicum experience took place at CellzDirect/Invitrogen Corporation (a part of Life Technologies) for 15 hours from August 27, 2008 to October 16, 2008 under the direction of Dr. Russell Thomas. During this time I assisted in conducting a metabolic stability analysis using cryopreserved human hepatocytes from two separate pools of ten individual donors. These pools were made from five male and five female donors. Incubation medium was prepared by supplementing William's E Medium with 0.1  $\mu\text{M}$  dexamethasone, 1 mL ITS+<sup>TM</sup> (per 500 mL medium), 4 mM Glutamax<sup>TM</sup>, 15 mM HEPES, and either 2  $\mu\text{g/mL}$  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, 2  $\mu\text{M}$  by adding 10  $\mu\text{L}$  1 mM chemical stock to 5 mL of incubation medium. Vials of cryopreserved hepatocytes were thawed using warm William's E Medium (37°C) and centrifuged at 76 X g for 6 minutes at 20°C. The cells were resuspended in incubation medium to a working cell density of  $1.0 \times 10^6$  viable cells per mL as determined by Trypan Blue<sup>TM</sup> exclusion. To each well of the incubation plates, 0.5 mL of  $1.0 \times 10^6$  viable cells per mL were added to yield a final cell density of  $0.5 \times 10^6$  viable cells per mL. Hepatocytes were plated and treated with 40 chemicals of environmental concern at 1 and 10  $\mu\text{M}$  concentrations. At time points of 0, 15, 30, 60, and 120 min, 50  $\mu\text{L}$  aliquots were removed from each well and quenched with 50  $\mu\text{L}$  ice cold acetonitrile. Duplicate

wells were run for each chemical. The quenched aliquots were frozen at  $-70^{\circ}\text{C}$  prior to analysis.

This study investigates the metabolic capacity of human hepatocytes when treated with different environmental chemicals. The residual concentrations of parent compound were determined by analytical chemistry and were plotted on a semi-log plot to generate a rate of metabolism. This data was then incorporated into a reverse pharmacokinetic model.

The second phase of my practicum research was performed in the lab at The Hamner Institutes for Health Sciences for 35 hours from July 14, 2009 to July 21, 2009 also under the direction of Dr. Russell Thomas, conducting a high throughput plasma protein binding experiment on 309 ToxCast phase I chemicals. The plasma was isolated from six individual donors using  $\text{K}_2\text{EDTA}$  as the anti-coagulant. The plasma was thawed at room temperature and centrifuged at  $2000 \times g$  for 10 min prior to analysis. Rapid equilibrium dialysis (RED) was conducted in 96-well format using the single-use RED plate according to manufacturer's instructions. Briefly, phosphate buffered saline (PBS) and plasma was loaded to the buffer and sample chambers, respectively, using a Biomek FX workstation. Chemical stock solutions were added to the sample chambers in triplicate to achieve final concentrations of 1 and  $10 \mu\text{M}$ . The concentration of DMSO did not exceed 0.5%. The RED plate was wrapped in aluminum foil and incubated at  $37^{\circ}\text{C}$  at 100 rpm in an orbital shaker for 4 hours. Aliquots were removed from the buffer and sample chambers, mixed with acetonitrile (100%) to precipitate proteins, and stored at  $-80^{\circ}\text{C}$  until analytical analysis.

This experiment yielded results for plasma protein binding affinity for 309 compounds which will be used in conjunction with measurements of metabolic clearance in a reverse pharmacokinetic model. This model will generate human oral equivalent dose values

for measurements of *in vitro* bioactivity. These data will provide the ability to use oral dose values which measure molecular perturbations to aid in the ToxCast's effort to perform chemical prioritization. There are an approximately 10,000 environmental chemicals which individuals may be exposed to. The vast majority of these chemicals have gone through limited or no toxicity testing (Judson et al., 2009). The current testing paradigm requires long term toxicity tests which require millions of dollars, many animal lives, and significant time investments. The ToxCast program is attempting to prioritize these chemicals for targeted toxicity testing and to reduce the overall costs of these tests. To accurately prioritize chemicals it is necessary to incorporate pharmacokinetics. An additional goal of the ToxCast program is to use the suite of *in vitro* assays and legacy *in vivo* rodent data to identify adverse toxicity pathways. The current regulatory methods use rodent *in vivo* data from frank toxicity observations to derive safe levels of chemicals. Oral equivalent values from *in vitro* assays that have been identified as representative of adverse effects could provide the ability of deriving a more accurate point of departure (POD) based on human modes of action. This approach could prove to be a major step towards basing toxicological regulatory decisions on human measurements from relevant exposure levels, eliminating the need for high dose animal studies for chemical safety testing.

The current regulatory methods are then analyzed along with estimates of human exposure based on crop application and water levels. These two assays will be used in a novel analysis that will provide a method of evaluation current regulatory standards and relate them to predicted levels of human bioactivity based on effects on human molecular targets. The methods described in this study contain the necessary elements to conduct a risk assessment analysis without the need for species extrapolation. This could have a large



impact on the amount of pesticides that farmers are allowed to spray on their crops. An alteration in the allowable levels for pesticide application could have a substantial economic impact as well as an impact on crop yields. It is important to understand the risk these chemicals pose for humans in order to protect not only the consumers of these products but the individuals responsible for the application of these chemicals.

## Appendix A

### Table of Estimated Exposure Values

Compound	CASRN	ePAD (mg/kg/day)	eRD (mg/kg/day)	% ePAD	% eRD	eMOE	Chronic Exposure Estimate (mg/kg/day)	Reference
2,4-D	94-75-7	0.005	0.005	38	NA	NA	0.0019	[U.S. EPA] U. S. Environmental Protection Agency. 2005. Reregistration eligibility decision for 2,4-D. U.S. Environmental Protection Agency, Washington, DC. p. 41 table 17 Web. April 7, 2010.
Acetochlor	34256-82-1	0.02	0.02	0.9	NA	NA	0.00018	[U.S. EPA] U. S. Environmental Protection Agency. 2006. Tolerance Reassessment Progress and Risk Management Decision (TRED) for Acetochlor. U.S. Environmental Protection Agency, Washington, DC. p. 4 Web. April 7, 2010.
Alachlor	25972-80-8	0.01	0.01	4	NA	NA	0.0004	[U.S. EPA] U. S. Environmental Protection Agency. 1998. Reregistration eligibility decision for Alachlor. U.S. Environmental Protection Agency, Washington, DC. p. 110 table 33. Web. April 7, 2010.
Atrazine	2912-24-9	0.0018	0.008	667	NA	NA	0.012066	[U.S. EPA] U. S. Environmental Protection Agency. 2008. Interim reregistration eligibility decision for Atrazine. U.S. Environmental Protection Agency, Washington, DC. p. 24 table 5. Web. April 7, 2010.
Benfloride	743-58-2	0.005	0.005	0.9	NA	NA	0.000045	[U.S. EPA] U. S. Environmental Protection Agency. 2000. Interim reregistration eligibility decision for Benfloride. U.S. Environmental Protection Agency, Washington, DC. p. 12. Web. April 7, 2010.
Bentazone	25057-89-0	0.003	0.03	28	NA	NA	0.00084	"Bentazon; Pesticide Tolerance, Final Rule." Federal Register 66 no. 180 [17 September 2001]: 48003-48011. table 3. Print.
Bisphenol-A	80-05-7	NA	0.05	NA	NA	NA	0.0000773*	[U.S. 1915] U. S. Department of Health and Human Services. 2008. NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol-A. U.S. Department of Health and Human Services, Washington, DC. p. 5 table 2. NIH Pub no. 08-5994.
Bromacil	314-80-9	NA	0.1	NA	1	NA	0.001	[U.S. EPA] U. S. Environmental Protection Agency. 1996. Reregistration eligibility decision for Bromacil. U.S. Environmental Protection Agency, Washington, DC. p. 20. Web. April 7, 2010.
Buprofezin	89327-76-0	0.0033	0.0033	80	NA	NA	0.00264	"Buprofezin; Pesticide Tolerance, Final Rule." Federal Register 74 no. 131 [10 July 2009]: p. 33157. Print.
Clothianidin	210880-92-5	0.0098	0.0098	16	NA	NA	0.001568	[U.S. EPA] U. S. Environmental Protection Agency. 2008. Dietary Exposure and Risk Assessment for Clothianidin. U.S. Environmental Protection Agency, Washington, DC. p. 7 table 3. Web. April 7, 2010.
Dichlorvos	62-73-7	0.0005	0.0005	NA	NA	39	0.001282051	[U.S. EPA] U. S. Environmental Protection Agency. 2006. Reregistration eligibility decision for Dichlorvos. U.S. Environmental Protection Agency, Washington, DC. p. 143. Web. April 7, 2010.
Dicofenfos	141-66-2	0.00002	0.00007	0.09	NA	NA	0.000000018	[U.S. EPA] U. S. Environmental Protection Agency. 2006. Reregistration eligibility decision for Dicofenfos. U.S. Environmental Protection Agency, Washington, DC. p. 11. Web. April 7, 2010.
Diuron	330-54-1	0.001	0.001	7	NA	NA	0.00021	[U.S. EPA] U. S. Environmental Protection Agency. 2003. Reregistration eligibility decision for Diuron. U.S. Environmental Protection Agency, Washington, DC. p. 18 table 6. Web. April 7, 2010.

Emamectin	155569-91-8	0.000075	0.000075	44	NA	NA	0.000033	"Emamectin; Pesticide Tolerance, Final Rule." Federal Register 74 no.11 (16 January 2009): 2871. Print. [U.S. EPA] U. S. Environmental Protection Agency. 2002 Interim Reregistration Eligibility Decision (IREED) for Fenamiphos . U.S. Environmental Protection Agency, Washington, DC. p.54. Web. April 7, 2010.
Fenamiphos	22224-90-6	0.0001	0.0001	0	NA	NA	0	"Fenamiphos; Pesticide Tolerance, Final Rule." Federal Register 69 no.189 (30 September 2004): 58320. Print. [U.S. EPA] U. S. Environmental Protection Agency. 2006 Reregistration eligibility decision for Fenamiphos . U.S. Environmental Protection Agency, Washington, DC. p.21 table 7. Web. April 7, 2010.
Fenprothion	68157-60-8	0.07	0.07	0.9	NA	NA	0.00063	"Fenprothion; Pesticide Tolerance, Final Rule." Federal Register 69 no.189 (30 September 2004): 58320. Print. [U.S. EPA] U. S. Environmental Protection Agency. 2006 Reregistration eligibility decision for Fenprothion . U.S. Environmental Protection Agency, Washington, DC. p.21 table 7. Web. April 7, 2010.
Methyl Parathion	298-00-0	0.00002	0.0002	8	NA	NA	0.0000206	"Methyl Parathion; Pesticide Tolerance, Final Rule." Federal Register 69 no.189 (30 September 2004): 58320. Print. [U.S. EPA] U. S. Environmental Protection Agency. 1998 Reregistration eligibility decision for Methyl Parathion . U.S. Environmental Protection Agency, Washington, DC. p.53. Web. April 7, 2010.
Metribuzin	21087-64-9	NA	0.013	NA	0.75	NA	0.00975	"Metribuzin; Pesticide Tolerance, Final Rule." Federal Register 69 no.189 (30 September 2004): 58320. Print. [U.S. EPA] U. S. Environmental Protection Agency. 2006 Reregistration eligibility decision for N-Octyl bicycloheptene dicarboximide (MGX-264) . U.S. Environmental Protection Agency, Washington, DC. p.35 table 11. Web. April 7, 2010.
MGX-264	113-48-4	0.061	0.061	51	NA	NA	0.03111	"MGX-264; Pesticide Tolerance, Final Rule." Federal Register 69 no.189 (30 September 2004): 58320. Print. [U.S. EPA] U. S. Environmental Protection Agency. 2006 Report of the Food Quality Protection Act (FQPA) Tolerance Reassessment Progress and Risk Management Decision (TRED) for Oxytetracycline . U.S. Environmental Protection Agency, Washington, DC. p.9 table 4. Web. April 7, 2010.
Oxytetracycline dihydrate	6153-64-6	0.0005	0.0005	95	NA	NA	0.000475	"Oxytetracycline; Pesticide Tolerance, Final Rule." Federal Register 67 no.233 (4 December 2002): 72208. Print. [U.S. EPA] U. S. Environmental Protection Agency. 2006 Reregistration eligibility decision for Propetamphos . U.S. Environmental Protection Agency, Washington, DC. p.11 table 3. Web. April 7, 2010.
Propetamphos	31218-83-4	0.0005	0.0005	23	NA	NA	0.000105	"Propetamphos; Pesticide Tolerance, Final Rule." Federal Register 67 no.233 (4 December 2002): 72208. Print. [U.S. EPA] U. S. Environmental Protection Agency. 2006 Reregistration eligibility decision for Triadimefon . U.S. Environmental Protection Agency, Washington, DC. p.17 table 3. Web. April 7, 2010.
Pyriproxyfen sodium	123343-16-8	0.0587	0.587	2	NA	NA	0.001174	"Pyriproxyfen Sodium; Pesticide Tolerance, Final Rule." Federal Register 67 no.233 (4 December 2002): 72208. Print. [U.S. EPA] U. S. Environmental Protection Agency. 2000 Reregistration eligibility decision for Vinclozolin . U.S. Environmental Protection Agency, Washington, DC. p.34 table 4. Web. April 7, 2010.
Triadimefon	43123-43-3	0.0034	NA	49	NA	NA	0.001666	"Triadimefon; Pesticide Tolerance, Final Rule." Federal Register 67 no.233 (4 December 2002): 72208. Print. [U.S. EPA] U. S. Environmental Protection Agency. 2008 Reregistration eligibility decision for Triclosan . U.S. Environmental Protection Agency, Washington, DC. p.17 table 3. Web. April 7, 2010.
Triclosan	3380-34-5	0.3	0.3	46.3	NA	290	0.1329	"Triclosan; Pesticide Tolerance, Final Rule." Federal Register 67 no.233 (4 December 2002): 72208. Print. [U.S. EPA] U. S. Environmental Protection Agency. 2000 Reregistration eligibility decision for Vinclozolin . U.S. Environmental Protection Agency, Washington, DC. p.34 table 4. Web. April 7, 2010.
Vinclozolin	50471-44-8	0.0012	0.012	7	NA	NA	0.000084	

\* Obtained by estimation of oral intake based on urinary output

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