Development of Reverse Toxicokinetic Models to Correlate *In Vitro* and *In Vivo* Estrogen Receptor Activity

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Abstract

High-throughput screening (HTS) assays provide an efficient way to identify chemicals with the potential to interfere with estrogen receptor (ER) pathways. However, nominal *in vitro* assay concentrations may not accurately reflect potential *in vivo* effects of these chemicals due to differences in bioavailability and clearance between the two systems. Therefore, we developed reverse toxicokinetic (TK) models to more accurately correlate *in vitro* concentrations with potential *in vivo* effects for two ER reference chemicals, 17β-estradiol (E2) and bisphenol A (BPA). Our TK models estimate the daily oral equivalent doses (OEDs) in laboratory animals and humans that would result in a steady-state *in vivo* blood concentration equivalent to the *in vitro* POD (point of departure) values from an ER-targeted HTS assay. We compared the estimated OEDs to human exposures and the *in vivo* dose range reported to elicit uterotrophic effects in laboratory animals. For both chemicals, we used published experimental data for hepatic clearance and unbound plasma protein fraction (Fub) to populate our models. We performed sensitivity analyses to evaluate the impact of both hepatic clearance and Fub on OED estimations. This modeling approach highlights the importance of TK considerations in ranking ER active chemicals based on *in vitro* HTS ER assays.

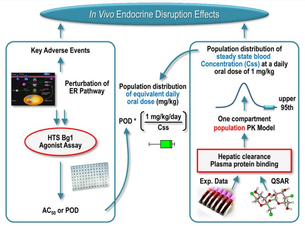
Introduction

- The U.S. Environmental Protection Agency (EPA) established the Endocrine Disruptor Screening Program (EDSP) in response to the 1996 Food Quality Protection Act (7 U.S.C. 136) and amendments to the Safe Drinking Water Act (110 Stat 1613). The laws required that the EPA screen pesticides and other chemicals for their potential for endocrine activity.
- As many as 10,000 chemicals may lack testing data to satisfy these requirements with several hundred new chemicals being produced each year (EPA 2011).
- The EDSP consists of a two-tiered screening and testing strategy including *in vivo* and *in vitro* tests. Under this strategy, EDSP testing could cost millions of dollars per chemical and take years to complete.
- Therefore, efforts are ongoing to establish high throughput screening (HTS) assays and *in silico* models to speed up the screening process.

Development of a Reverse Toxicokinetic Model for Estrogenic Effects

- The EDSP includes assays that assess chemical effects on estrogen signaling. Estrogen signaling is well-characterized and a number of test methods exist that target estrogenic pathways.
- One of these is the *in vitro* BG1Luc estrogen receptor (ER) transactivation assay (BG1Luc), which is accepted internationally for identifying ER agonists and has been adapted to an HTS format (BG1Luc HTS).
- Differences in bioavailability and clearance between *in vitro* and *in vivo* systems make it difficult to directly correlate test chemical concentration in an *in vitro* assay with the *in vivo* dose that could cause toxic effects. Extrapolation from *in vitro* to *in vivo* results must account for these differences and consider which pharmacokinetic (PK) factors (such as bioavailability, clearance, and protein binding) are relevant.
- The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) applied a population-based reverse toxicokinetic (TK) model to address this problem. The model correlates point of departure (POD) in the *in vitro* BG1Luc HTS assay to the lowest effective dose in the *in vivo* rat uterotrophic assay for two ER agonist reference chemicals with different estrogenic potency: 17β-estradiol (E2) and bisphenol A (BPA). BPA is generally regarded to be 10,000 to 100,000 times less potent than E2.
- The rationale for the model is illustrated in **Figure 1**.

Figure 1. Use of Pharmacokinetics Modeling for Reverse Dosimetry[1]



Abbreviations: AC_{50} = half-maximal activity concentration; C_{ss} = steady-state blood concentration; ER = estrogen receptor; Exp. = experimental; HTS = high-throughput screening; PK = pharmacokinetic; POD = point of departure; QSAR = quantitative structure-activity relationship.

[1] Adapted from Judson et al. (2011)

Data Used in the Analysis

- BPA and E2 were tested in the BG1Luc HTS agonist assay by the U.S. Tox21 screening program. Both substances were tested at 15 concentrations ranging from 2 nM to 100 µM.
- A number of literature reports provided data for tests of BPA and E2 in the rat uterotrophic assay (Ashby and Tinwell 1998; Diel et al. 2000; Matthews et al. 2001; Odum et al. 1997; Stroheker et al. 2003; Zhang et al. 2012). The dataset for this analysis included studies using an oral exposure route and either immature or ovariectomized rats.
- Reference values for the fraction of unbound plasma protein (Fub), *in vitro* hepatocyte metabolic clearance rates (CLinvitro), and *in vivo* intrinsic metabolic clearance rates (CLintrinsic) for BPA and E2 were obtained from the literature (Plowchalk and Teeguarden 2002; Teeguarden et al. 2005; Wetmore et al. 2012, 2013).
- Human exposure levels for BPA were based on an analysis of likely exposure pathways (Vandenberg et al. 2007), while exposure levels for E2 were based on doses of estrogenic compounds in oral contraceptive pills (Burkman et al. 2011).

In Vitro to *In Vivo* Dose Correlation

- The point of departure (POD) was defined as the lowest concentration that causes a response that significantly exceeds the background activity level in the assay.
- The daily oral equivalent doses (OEDs) that would result in median *in vivo* steady-state blood concentrations (Css) equal to the POD were estimated using reverse PK models for rat and human (**Figure 1**). The OEDs were then compared to (a) the lowest oral dose that resulted in a significant increase in rat uterine weight or (b) human exposure data.
  - A simple one-compartment population PK model was used to estimate median Css, which assumes 100% oral absorption and includes both renal and hepatic metabolic clearances.
  - The standard Css in the blood for a daily oral dose of 1 mg/kg/day in rat is calculated as:

$$Css\ (\mu M) = \frac{\frac{1\ ((mg/kg)/d)}{24} * BW(kg)}{CLrenal(l/h) + CLhepatic(l/h)} * 1000/MW$$

In which:

$$CLrenal\ (l/h) = GFR(l/h) * Fub$$

$$CLhepatic\ (l/h) = Qliver\ (l/h) * \frac{Fub * CLintrinsic}{Qliver + Fub * CLintrinsic}$$

$$CLintrinsic = CLinvitro * \#\ of\ Heptocytes\ /gram\ liver * Liver\ weight$$

  - The standard Css was then used to calculate the OED that results in a median Css equivalent to the POD from the BG1Luc HTS agonist assay for a given chemical (Wetmore et al. 2013):

$$Oral\ Equivalent\ Dose\ (mg/kg/d) = POD * 1\ (mg/kg/d)/Css$$

- To evaluate and better understand the impact of Fub and CLinvitro on rat OEDs for BPA and E2, we systematically examined the effects of both varying Fub from 0.000 to 1 and varying CLinvitro from 0.1–100 µl/min per million rat hepatocytes.

Results

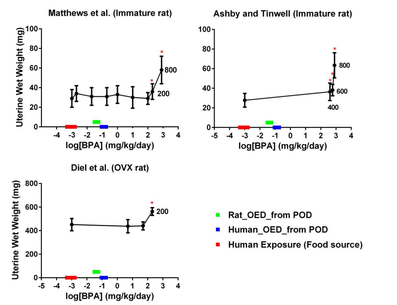
- Both BPA and E2 are strongly bound to plasma protein in rat and human (**Table 1**).
  - The hepatic clearance rate of BPA in rat is about one-fifth that of E2 (CLinvitro: 2.33 for BPA versus 14.99 for E2).
  - The clearance rate for the two substances is not significantly different in human (CLinviro: 19.3 for BPA versus 14.2 for E2).
- For BPA, the rat OED estimated from the BG1 POD was over 1000 times less than the lowest effective oral dose in three separate rat uterotrophic assay studies, while the human OED estimated from the BG1 POD is about 100 times more than the general human exposure to BPA through food sources (**Figure 2**).
- For E2, the rat OED estimated from the BG1 POD was 4–76 times less than the lowest effective oral dose in three separate rat uterotrophic assay studies, while the human OED estimated from the BG1 POD is comparable to the human exposure to E2 via oral contraceptive pills (**Figure 3**).
- The POD for BPA in the BG1Luc HTS agonist assay is about 147 times that of E2, while the OED estimated for BPA is about 32 times higher than that of E2 in rat and nearly 500 times that of E2 in human, suggesting an *in vitro* to *in vivo* potency shift after considerations of dosimetry and PK factors by species (**Table 2**).
- For both chemicals, fluctuations in OED estimations are directly proportional to the CLinvitro and Fub (**Figure 4**, **Tables 3** and **4**).

Table 1. Physiological and Biochemical Parameters and *In Vitro* Assay Inputs Used in the Population PK Model

	Physiological Parameter		Biochemical Parameter				BG1Luc HTS Agonist Assay
Compound_ Species	GFR (l/h)	Qliver (l/h)	Fub	CLinvitro (µl/min/ 10^6 cells)	CLintrinsic (l/h)	CLhepatic (l/h)	POD (µM)
BPA_Rat	0.08	0.83	0.06	2.33	0.16	0.01	0.14
E2_Rat			0.05	14.99	1.00	0.05	0.00093
BPA_Human	6.7	90	0.07	19.29	203.23	12.01	0.14
E2_Human			0.02	14.24	150.00	2.76	0.00093

Abbreviations: BPA = bisphenol A; CLhepatic = hepatic clearance rate; CLintrinsic = intrinsic metabolic clearance rate; CLinvitro = *in vitro* hepatocyte metabolic clearance rate; E2 = 17β- estradiol; Fub = fraction of unbound plasma protein; GFR = glomerular filtration rate; HTS = high throughput screening; Qliver = liver blood flow; POD = point of departure.

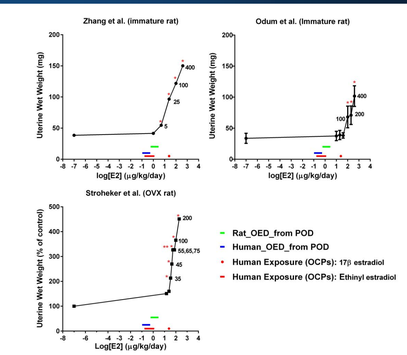
Figure 2. Uterotrophic Data, Estimated OEDs, and Estimated Human Exposure for BPA



Abbreviations: BPA = bisphenol A; OED = daily oral equivalent doses; OVX = ovariectomized; POD = point of departure.

Line graphs represent rat uterotrophic data from three separate studies (Ashby and Tinwell 1998; Diel et al. 2000; Matthews et al. 2001). Red asterisks indicate values that are significantly different from control ($p < 0.05$). The red bar along each horizontal axis represents estimates human exposure to BPA from food sources (Vandenberg et al. 2007). The other colored bars along each horizontal axis represent OEDs estimated from the BG1Luc HTS POD using the rat or human population PK models.

Figure 3. Uterotrophic Data, Estimated OEDs, and Estimated Human Exposure for E2



Abbreviations: E2 = β-estradiol; OED = daily oral equivalent doses; OCPs = oral contraceptive pills; OVX = ovariectomized; POD = point of departure.

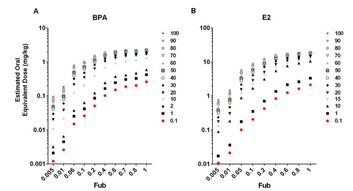
Line graphs represent rat uterotrophic data from three separate studies (Odum et al. 1997; Stroheker et al. 2003; Zhang et al. 2012). Red asterisks indicate values that are significantly different from control ($p < 0.05$). The red dot and bar along each horizontal axis represent human exposure to 17β-estradiol and ethinyl estradiol, respectively, from birth control pills. The other colored bars along each horizontal axis represent OEDs estimated from the BG1Luc HTS POD using the rat or human population PK models.

Table 2. Comparison of BPA and E2 OED by Species

Compound and Species	POD (µM) From BG1Luc HTS Agonist Assay	OED (mg/kg) Estimated From POD
BPA_Rat	0.14	0.0423
BPA_Human		0.1269
E2_Rat	0.00093	0.0013
E2_Human		0.00026
Ratio (BPA/E2)	**146.6**	**31.8 (Rat), 497.6 (Human)**

Abbreviations: BPA = bisphenol A; E2 = 17β- estradiol; HTS = high throughput screening; OED = daily oral equivalent dose; POD = point of departure.

Figure 4. Impact of CLinvitro and Fub on OED Estimated From BG1Luc HTS POD for BPA and E2 in Rat



Abbreviations: BPA = bisphenol A; E2 = 17β- estradiol; CLinvitro = *in vitro* hepatocyte metabolic clearance rate; Fub = fraction of unbound plasma protein; HTS = high throughput screening; OED = daily oral equivalent dose; POD = point of departure.

Each symbol represents a different value of CLinvitro in units of µl/min per million rat hepatocytes. The values for each data point are contained in **Table 3** (for BPA) and **Table 4** (for E2).

Table 3. Effect of Varying Fub or CLinvitro on OED Estimated From BG1Luc HTS POD for BPA in Rat

		Estimated OEDs												
		CLinvitro (µl/min per million rat hepatocytes)												
		0.1	1	2	10	20	30	40	50	60	70	80	90	100
Fub	0.005	0.001	0.002	0.003	0.01	0.02	0.03	0.04	0.05	0.06	0.06	0.08	0.09	0.10
	0.01	0.003	0.004	0.01	0.02	0.04	0.06	0.08	0.10	0.11	0.13	0.15	0.16	0.19
	0.06	0.02	0.03	0.04	0.13	0.22	0.32	0.40	0.49	0.56	0.64	0.69	0.74	0.80
	0.1	0.03	0.04	0.06	0.20	0.35	0.48	0.62	0.71	0.81	0.86	0.94	1.01	1.05
	0.2	0.05	0.09	0.12	0.40	0.63	0.82	0.99	1.13	1.20	1.35	1.40	1.47	1.54
	0.4	0.10	0.17	0.25	0.68	1.04	1.25	1.43	1.56	1.68	1.75	1.81	1.90	1.93
	0.6	0.15	0.25	0.37	0.91	1.31	1.56	1.73	1.83	1.92	2.00	2.02	2.10	2.15
	0.7	0.19	0.31	0.41	1.05	1.47	1.71	1.84	2.02	2.05	2.12	2.17	2.20	2.26
	0.8	0.21	0.33	0.47	1.12	1.57	1.77	1.92	2.02	2.07	2.13	2.23	2.28	2.27
	1	0.26	0.43	0.60	1.31	1.69	1.95	2.06	2.16	2.26	2.27	2.32	2.37	2.38

Abbreviations: BPA = bisphenol A; CLinvitro = *in vitro* hepatocyte metabolic clearance rate; Fub = fraction of unbound plasma protein; HTS = high throughput screening; OED = daily oral equivalent dose; POD = point of departure.

OED estimated using experimental Fub and CLinvitro for BPA; OED estimation within 2-fold of the value highlighted in yellow; OED estimation within 5-fold of the value highlighted in yellow.

Table 4. Effect of Varying Fub or CLinvitro on OED Estimated From BG1Luc HTS POD for E2 in Rat

		Estimated OEDs												
		CLinvitro (µl/min per million rat hepatocytes)												
		0.1	1	2	10	20	30	40	50	60	70	80	90	100
Fub	0.005	0.01	0.02	0.09	0.13	0.18	0.24	0.31	0.38	0.47	0.54	0.64	0.68	0.77
	0.01	0.02	0.04	0.18	0.17	0.31	0.47	0.63	0.78	0.95	1.09	1.19	1.37	1.48
	0.1	0.10	0.18	0.88	1.20	1.56	2.17	2.77	3.31	3.94	4.36	4.94	5.54	5.86
	0.2	0.21	0.35	1.70	2.27	2.89	4.09	5.04	5.79	6.67	7.29	7.90	8.74	8.96
	0.4	0.43	0.71	3.14	4.28	5.02	7.17	8.15	9.03	10.01	10.78	11.28	11.99	12.69
	0.6	0.85	1.36	5.48	7.41	8.54	10.51	12.13	12.91	13.79	14.70	14.81	15.48	16.29
	0.7	1.25	2.17	7.73	9.63	10.86	13.12	14.49	15.19	16.12	16.47	16.99	17.46	17.81
	0.8	1.64	2.62	9.25	11.10	12.31	14.33	15.84	16.78	17.42	17.62	18.26	18.54	18.70
	1	2.14	3.35	10.65	12.75	14.31	15.74	16.97	17.74	18.45	18.52	19.38	19.48	19.57

Abbreviations: CLinvitro = *in vitro* hepatocyte metabolic clearance rate; E2 = 17β- estradiol; Fub = fraction of unbound plasma protein; HTS = high throughput screening; OED = daily oral equivalent dose; POD = point of departure.

OED estimated using experimental Fub and CLinvitro for E2; OED estimation within 2-fold of the value highlighted in yellow; OED estimation within 5-fold of the value highlighted in yellow.

Discussion

- Compared to the lowest effective dose levels in the *in vivo* rat uterotrophic assay, the oral equivalent doses predicted from the reverse TK model using the POD of the *in vitro* BG1Luc HTS agonist assay are lower for both chemicals, suggesting that the *in vitro* assay provides a more conservative hazard estimate.
- Differences between the PODs for the BG1 agonist assay and the rat OEDs for BPA and E2 confirm that PK factors need to be integrated when applying the nominal effective concentration from *in vitro* assay to risk assessment.
- Fluctuations in OED estimations are directly proportional to CLinvitro and Fub. The overall impact is less for E2 than for BPA, indicating that this effect varies with the specific chemical being considered.

Conclusions

- The nominal effective concentration in the *in vitro* assay should be adjusted for important toxicokinetic factors to predict *in vivo* effects.
- Compared to the *in vivo* rat uterotrophic assay, the *in vitro* BG1 HTS agonist assay provides a more conservative estimate for use in risk assessment.
- The effect of variations in Fub and CLinvitro on the overall *in vitro* to *in vivo* extrapolation is chemical dependent.

Future Directions

- NICEATM is applying this reverse TK modeling approach to other EDSP reference compounds to improve *in vitro* to *in vivo* prediction on effects that occur through the ER-mediated pathway.
- Reverse TK modeling will be an important component of future efforts to link *in vitro* assays and *in vivo* endpoints for various toxicities and adverse outcome pathways.

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