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

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Abstract

Through the U.S. Tox21 high-throughput screening (HTS) program, efforts are underway to use quantitative high-throughput *in vitro* assays to assess chemical effects across multiple cellular pathways, including the estrogen receptor (ER) pathway. HTS assays provide an efficient way of identifying potential biological targets for chemicals. However, the nominal *in vitro* assay concentrations may not accurately reflect the effective *in vivo* doses of these chemicals due to the differences in bioavailability and clearance. A set of pharmacokinetic models was developed to correlate *in vitro* concentration with effective *in vivo* dose for Tox21 chemicals with potential to interact with the ER. These models estimate the daily oral equivalent doses (OEDs) in laboratory animals and humans for Tox21 ER active chemicals that would result in a steady-state *in vivo* blood concentration equivalent to the *in vitro* POD (point of departure) values identified using HTS assays that specifically target the ER pathway. These models were built using published experimental data and quantitative structure–activity relationship predictions for hepatic metabolic clearance and unbound plasma protein fraction. The models were also adapted to incorporate infant physiology to include this most vulnerable human population. Using OEDs estimated from the model, Tox21 ER active chemicals were ranked, with chemicals having the lowest effective dose in these models being considered the most likely to interact with the ER *in vivo*, either as agonists or antagonists. The estimated oral dose for a subset of chemicals was also compared to the *in vivo* dose range reported to elicit ER-related effects.

Introduction

- U.S. (7 U.S.C. 136, 110 Stat 1613) and international regulations require the testing of chemicals for the detection of potential 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 U.S. Environmental Protection Agency established the Endocrine Disruptor Screening Program (EDSP) to address these requirements. The EDSP uses a two-tiered strategy for identification of endocrine active chemicals. First tier testing consists of *in vitro* and *in vivo* screens, which could ultimately cost millions of dollars per chemical, take years to complete, and utilize many animals.
- Therefore, efforts are ongoing within the Tox21 Program to establish a testing strategy based on *in vitro* high throughput screening (HTS) assays and *in silico* models that could 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 the effective test chemical concentration in an *in vitro* assay with the *in vivo* dose that could cause biological/toxic effects. Extrapolation from *in vitro* to *in vivo* results must account for these differences and consider which pharmacokinetic (PK) factors are most relevant.
- To address this issue, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) developed a population-based reverse toxicokinetic (TK) model. The model (**Figure 1**) correlates point of departure (POD) in the *in vitro* BG1Luc HTS assay to the lowest effective dose (LED) in the rat uterotrophic assay or daily human exposure for selected Tox21 chemicals. The model was first tested using two ER agonist reference chemicals with different estrogenic potency (17β-estradiol [E2] and bisphenol A [BPA]). The model was then expanded to include 24 more Tox21 compounds (listed in **Tables 5** and **6**).
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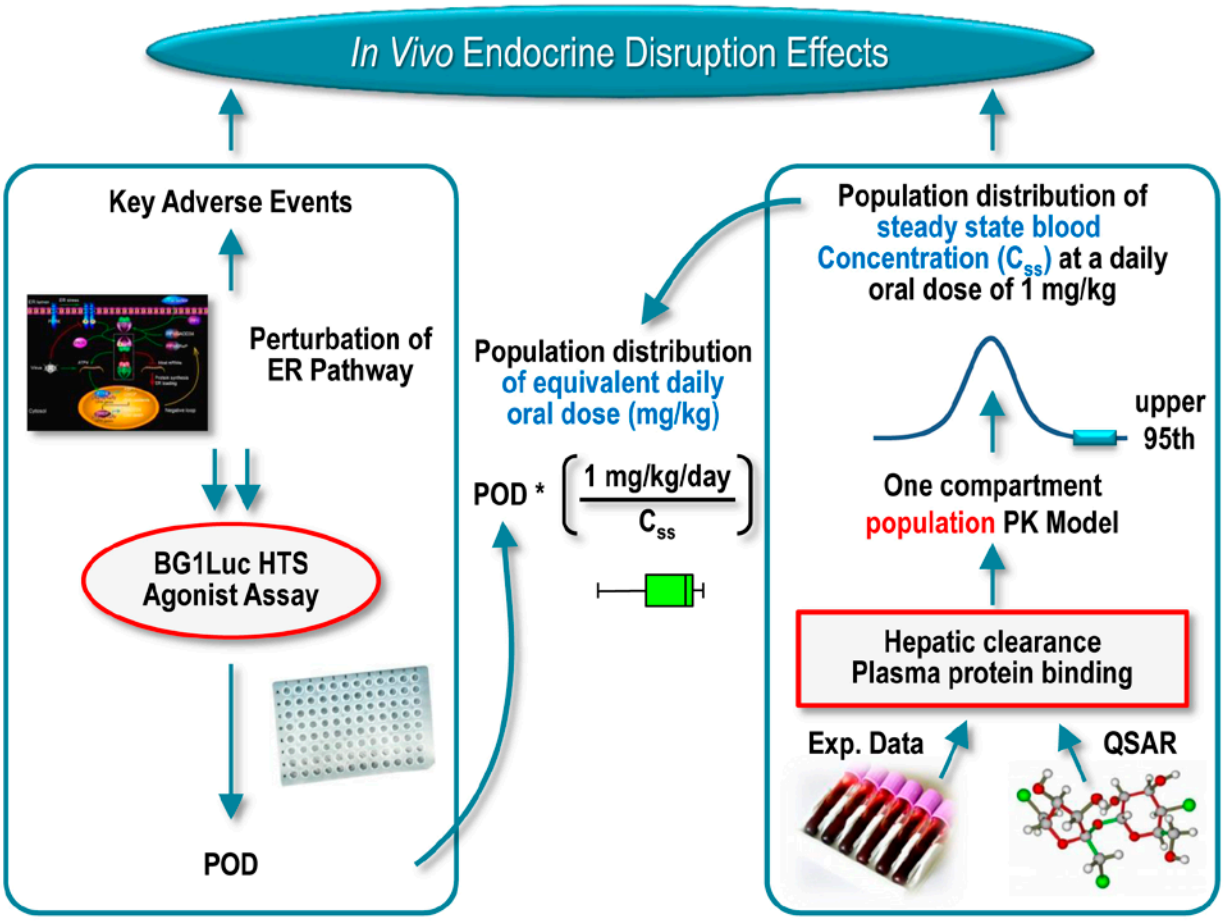
Figure 1. Use of Pharmacokinetics Modeling for Reverse Dosimetry[1]



Abbreviations: Css = 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

- Each of the chemicals included in this evaluation was tested in the BG1Luc HTS agonist assay by the U.S. Tox21 screening program at 15 concentrations ranging from 2 nM to 100 µM.
- Rat uterotrophic data were obtained from the scientific literature (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 in either immature or ovariectomized rats with mainly oral exposure routes.
- Most of the reference values for the fraction of unbound plasma protein (Fub), *in vitro* hepatocyte metabolic clearance rates (CLinvitro), and *in vivo* intrinsic metabolic clearance rate (CLintrinsic) were obtained from the literature (Plowchalk and Teeguarden 2002; Teeguarden et al. 2005; Wetmore et al. 2012, 2013). Fub values were predicted using a commercially available structural–activity relationship model (Simulation Plus, Inc.) for chemicals for which such data were not available.
- 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). U.S. population exposure estimates for other Tox21 chemical were obtained from the scientific literature (Wetmore et al. 2012).

In Vitro to *In Vivo* Dose Correlation

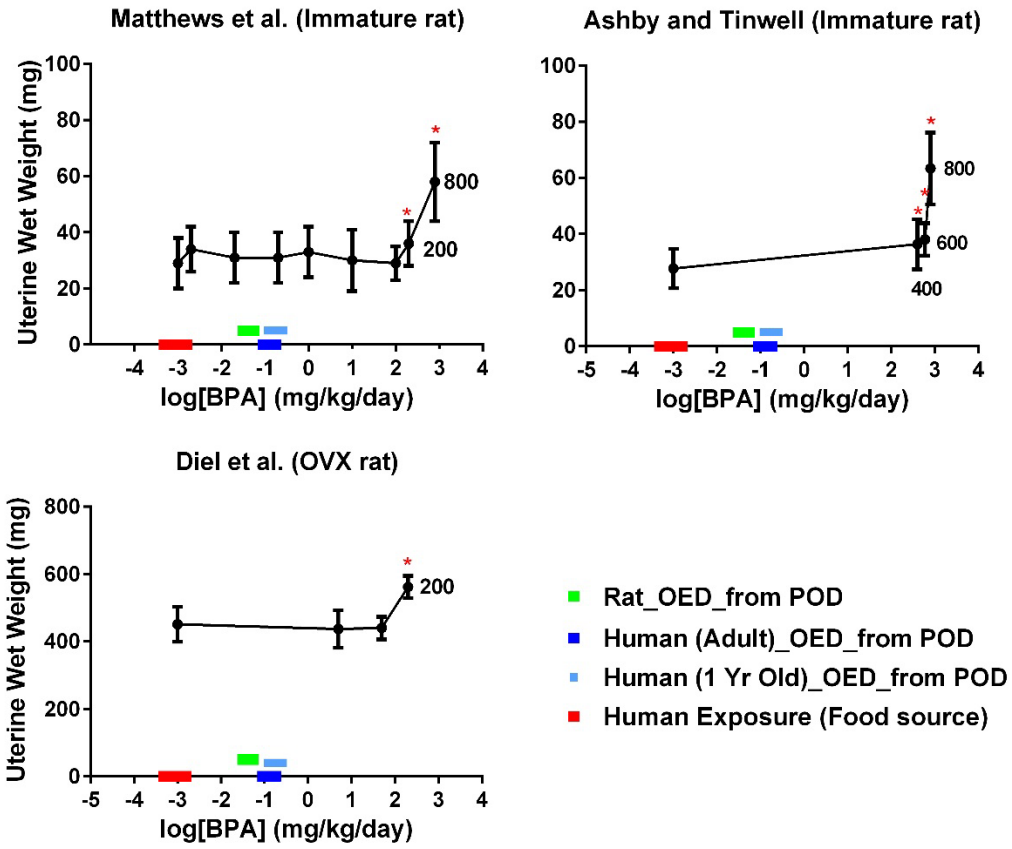
- The point of departure (POD) was defined as the lowest concentration that causes a response that significantly exceeds the background activity level in the BG1Luc HTS assay.
- The daily oral equivalent doses (OEDs) that would result in median *in vivo* steady-state blood concentrations (Css) equal to the POD were estimated using reverse TK models for rat, human adult, and human infant (assumed to be one year old) (**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 for a daily oral dose of 1 mg/kg/day is calculated as:

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	0.060	2.33	0.16	0.01	0.14
E2_Rat			0.053	14.99	1.00	0.05	0.00093
BPA_Human (Adult)	6.7	90	0.068	19.29	203.23	12.01	0.14
E2_Human (Adult)			0.019	14.24	150.00	2.76	0.00093
BPA_Human (Infant)			0.076	19.29	38.2	2.55	0.14
E2_Human (Infant)	1.8	21.4	0.021	14.24	28.2	0.59	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.

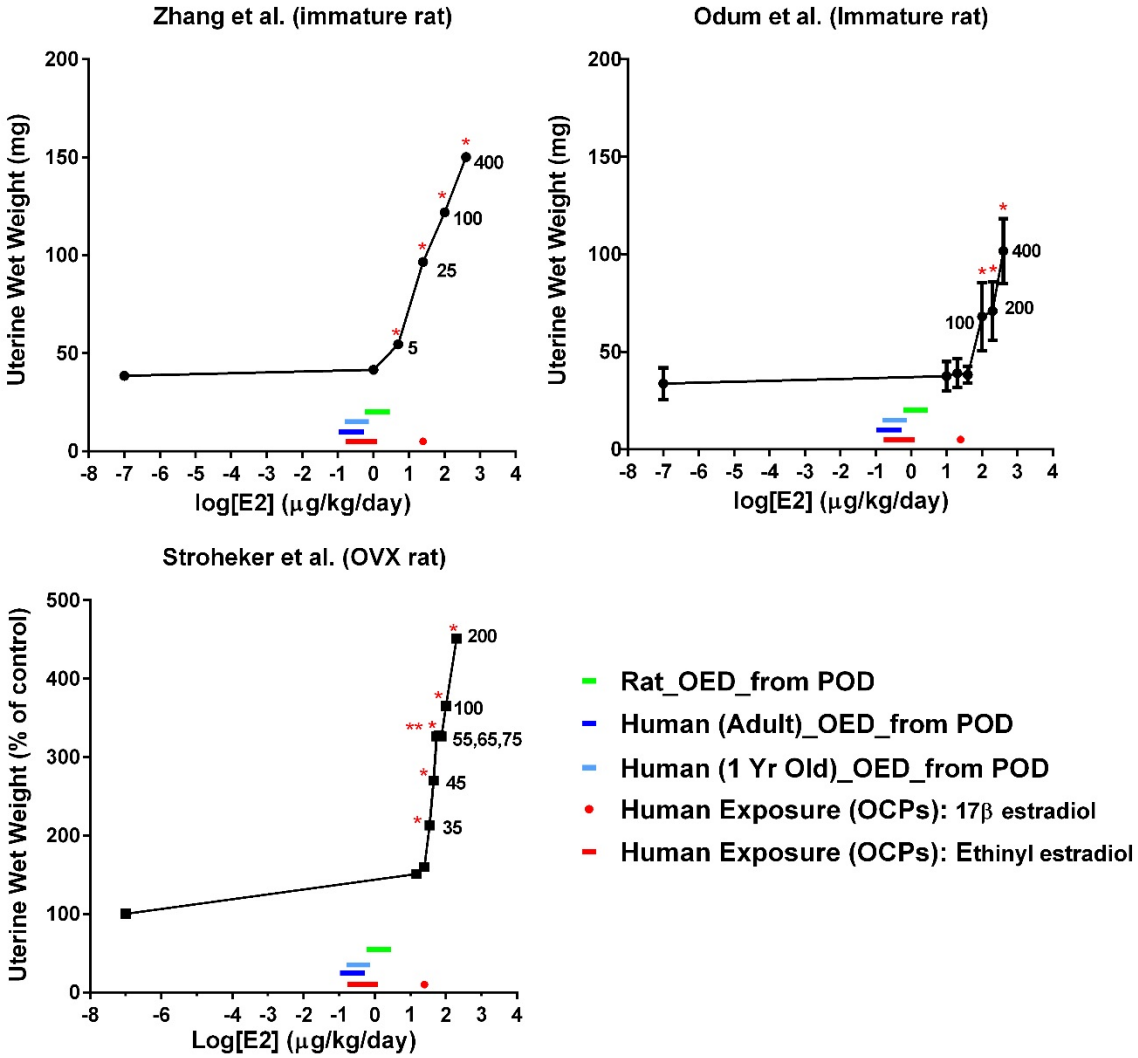
Figure 2. Uterotrophic Data, Estimated OEDs, and Estimated Human Exposure for BPA



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 estimated 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.

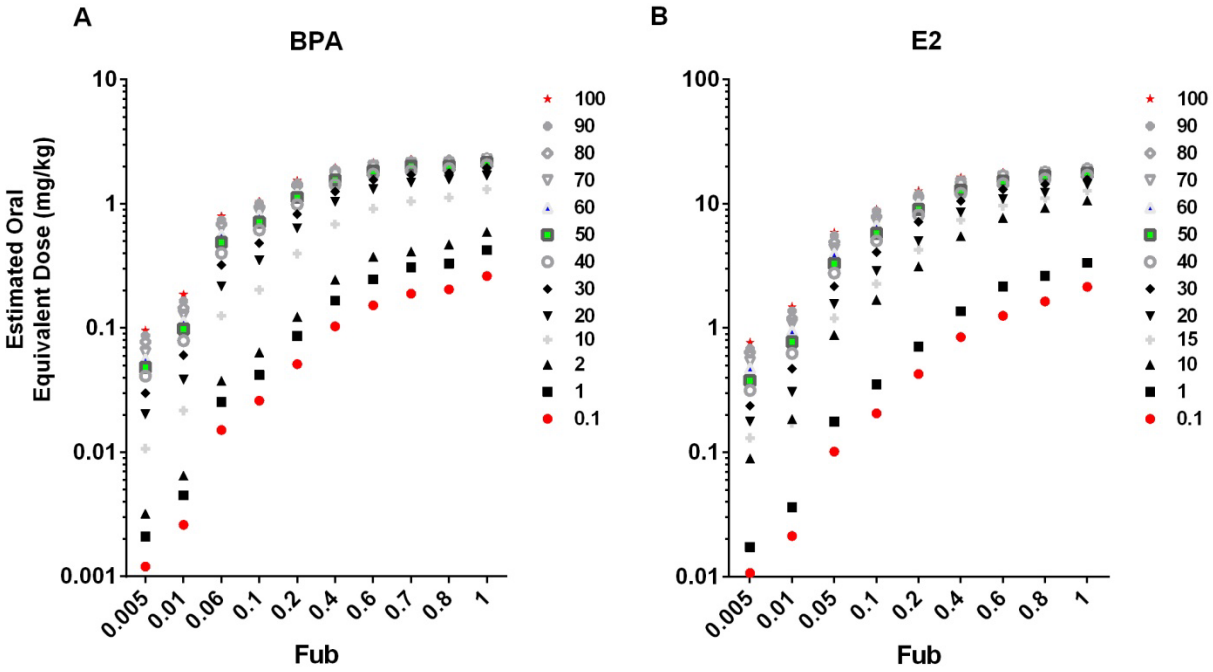
Line graphs represent rat uterotrophic data from three separate studies (Odum et al. 1997; Stroheker et al. 2003; Zhang et al. 2012). Red asterisks indicate values that are significantly different from control ($p < 0.05$). The red dot and bar along each horizontal axis represent human exposure to 17β-estradiol 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	Median OED (mg/kg/day) Estimated from POD
BPA_Rat	0.14	0.042
BPA_Human (Adult, Infant)		0.1269, 0.1774
E2_Rat	0.00093	0.0013
E2_Human (Adult, Infant)		0.00026, 0.00034
Ratio (BPA/E2)	**146.6**	**31.8 (Rat), 497.6 (Human Adult), 525.8 (Human Infant)**

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 Estimation from BG1Luc HTS POD for BPA and E2 in Rat



Abbreviations: BPA = bisphenol A; 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.

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 Estimates from BG1Luc HTS POD for BPA in Rat

Estimated OEDs														
		CL invitro (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 estimate within 2-fold of the value highlighted in yellow; OED estimate within 5-fold of the value highlighted in yellow.

Table 4 Effect of Varying Fub or CLinvitro on OED Estimates from BG1luc HTS POD for E2 in Rat

Estimated OEDs														
		CL invitro (µl/min per million rat hepatocytes)												
		0.1	1	10	15	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
	0.05	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
	0.1	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
	0.2	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
	0.4	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
	0.6	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 estimate within 2-fold of the value highlighted in yellow; OED estimate within 5-fold of the value highlighted in yellow.

Table 5 Selected Tox21 Chemicals with OEDs for Rat Estimated from *In Vitro* Assay Endpoints and LEDs from Uterotrophic Assays

Chemical[1]	R1 Score[2]	POD (µM) from BG1Luc HTS Agonist Assay	Median OED (mg/kg/day)	LED from Uterotrophic Assay (mg/kg/day)	Ratio: LED/OED
Bisphenol A	0.458	0.137	0.042	200	4755
Fenarimol	0.012	10.233	1.585	200	126
Lindane	0.001	NEG	NA	NEG	NA
Acetochlor	0	NEG	NA	NEG	NA
Diethyhexyl phthalate	0	NEG	NA	NEG	NA
Fenbuconazole	0	NEG	NA	NEG	NA
Perfluorooctanoic acid	0	NEG	NA	NEG	NA
Permethrin[3]	0	NEG	NA	800	NA
Simazine	0	NEG	NA	NEG	NA
Triclosan	0	NEG	NA	NEG	NA

Abbreviations: HTS = high-throughput screening; LED = lowest effective dose; NA = not applicable; NEG = negative; OED = daily oral equivalent dose; POD = point of departure.

[1] Compounds are sorted by increasing median OED and then alphabetically by compound name for those that had R1 scores of zero.

[2] R1 score is a concatenated number assigned to each chemical based on an ER pathway model (Judson et al. in preparation). An R1 score greater than zero indicates that a chemical is a potential ER agonist.

[3] For permethrin, there was a discrepancy between the ER pathway model prediction and the uterotrophic assay outcome (yellow highlight).

Table 6 Selected Tox21 Chemicals with OEDs for Adult Humans Estimated from *In Vitro* Assay Endpoints and Estimated Human Exposure

Chemical[1]	R1 Score[3]	POD (µM) from BG1Luc HTS Agonist Assay	Median OED (mg/kg/day)	Human Exposure Estimates (mg/kg/day)	Ratio: OED/Highest Exposure Reported
2,2-Bis(4-hydroxyphenyl)-1,1,1-trichloroethane	0.612	0.02	0.015	NA	NA
Endosulfan	0.042	2.19	0.083	0.000006 -- 0.000102	816.18
Bisphenol A	0.682	0.14	0.121	0.00048 -- 0.0016	75.47
Fenarimol	0.061	10.23	0.289	<0.00002	>14442.32
Pendimethalin	0.010	17.38	1.070	0.00042 -- 0.0014	763.98
Methoxychlor	0.373	3.44	3.259	NA	NA
Dibutyl phthalate	0.089	18.20	8.974	NA	NA
Benomyl[3]	0	40.74	13.412	NA	NA
Acetochlor	0	NEG	NA	0.0004 -- 0.0012	NA
Atrazine	0	NEG	NA	NA	NA
Cyanazine	0	NEG	NA	NA	NA
Diethyhexyl phthalate	0	NEG	NA	0.0630	NA
Esfenvalerate	0	NEG	NA	0.000058 -- 0.000134	NA
Fenbuconazole	0	NEG	NA	0.00044 -- 0.002027	NA
Lindane[3]	0.003	NEG	NA	NA	NA
Metolachlor	0	NEG	NA	0.000199 -- 0.000641	NA
Monobutyl phthalate	0	NEG	NA	NA	NA
Pentachloronitrobenzene	0	NEG	NA	0.000194 -- 0.001597	NA
Perfluorooctanoic acid	0	NEG	NA	NA	NA
Permethrin	0	NEG	NA	0.000184 -- 0.000432	NA
Simazine	0	NEG	NA	NA	NA
Tetramethrin	0	NEG	NA	NA	NA
Triclosan	0	NEG	NA	0.0293 -- 0.13498	NA
Vinclozolin	0	NEG	NA	0.000034 -- 0.000078	NA

Abbreviations: HTS = high-throughput screening; LED = lowest effective dose; NA = not applicable; NEG = negative; OED = daily oral equivalent dose; POD = point of departure.

[1] Compounds are sorted by increasing median OED and then alphabetically by compound name for those that have no median OED.

[2] R1 score is a concatenated number assigned to each chemical based on an ER pathway model (Judson et al. in preparation). An R1 score greater than zero indicates that a chemical is a potential ER agonist.

[3] For benomyl and lindane, there was a discrepancy between ER pathway model prediction (yellow highlight) and *in vitro* assay result.

Discussion

- The OEDs derived from the BG1 Luc HTS assay are lower than the LEDs in the rat uterotrophic assay, suggesting the *in vitro* data provide a more conservative hazard estimate. Ratio differences among the PODs from the BG1 agonist assay, rat OEDs, and human OEDs between BPA and E2 confirm that PK factors need to be integrated when applying the nominal effective concentration from *in vitro* assays to risk assessment.
- OED estimates vary depending on the accuracy of experimentally derived values for CLinvitro and Fub. The extent of this effect varies depending on the specific chemical. However, our data for E2 and BPA indicate that experimental values for Fub and CLinvitro can vary up to 5-10 fold without significantly impacting the overall OED estimate.
- The incorporation of dosimetry, species- or age- specific toxicokinetics, and exposure is necessary for proper interpretation of *in vitro* HTS ER assay data for risk assessments. A good concordance between *in vitro* and *in vivo* endpoints provide confidence in using the BG1Luc HTS assay to speed up the screening process for potential endocrine disrupting compounds.

Conclusion

- The nominal effective concentration in the *in vitro* assay should be adjusted for important toxicokinectic factors to more accurately predict *in vivo* effects.
- Compared to the *in vivo* rat uterotrophic assay, the *in vitro* BG1 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

- 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.
- NICEATM is applying this reverse TK modeling approach to a broader set of EDSP reference compounds to optimize the *in vitro* to *in vivo* prediction of effects that occur through the ER pathway.

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