

## Application of Reverse Dosimetry to Compare *In Vitro* and *In Vivo* Estrogen Receptor Activity

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### Abstract

*In vitro* assays provide an efficient way to identify endocrine-active chemicals. However, nominal *in vitro* assay concentrations of a chemical may not accurately reflect the blood or tissue levels that cause *in vivo* effects, mostly due to differences in bioavailability and clearance between the two systems. In this study, we developed and applied pharmacokinetic (PK) and physiologically based pharmacokinetic (PBPK) models to quantitatively correlate *in vitro* and *in vivo* dosimetry for estrogen receptor (ER) reference chemicals. All the chemicals were tested in an estrogen receptor transactivation assay, BG1Luc, from which we derived point-of-departure (POD) values for each chemical. Using these PK/PBPK models, we estimated the injection or oral daily equivalent doses (IEDs or OEDs) that would result in a steady-state blood concentration (C<sub>ss</sub>) or maximum blood concentration (C<sub>max</sub>) value equivalent to the POD values. Critical model parameters (e.g. metabolic clearance, fraction of plasma protein binding) were derived from published experimental data or predicted from quantitative structure–activity relationship models. Where available, the daily IEDs or OEDs were compared to the lowest effective levels (LELs) in rat uterotrophic assays with corresponding administration routes. Our preliminary results showed that OED estimated using BG1Luc assay data for bisphenol A, a highly studied and environmentally relevant ER reference chemical, was lower than the lowest oral LEL for this chemical in rat uterotrophic assays, suggesting that the BG1Luc assay may provide a more conservative hazard estimate for use in risk assessment. Our modeling approach highlights the importance of pharmacokinetic considerations in assessing and ranking endocrine-active chemicals based on *in vitro* assays. (This abstract differs slightly from the published version: it was revised to reflect the content of the poster, which contains more current data.)

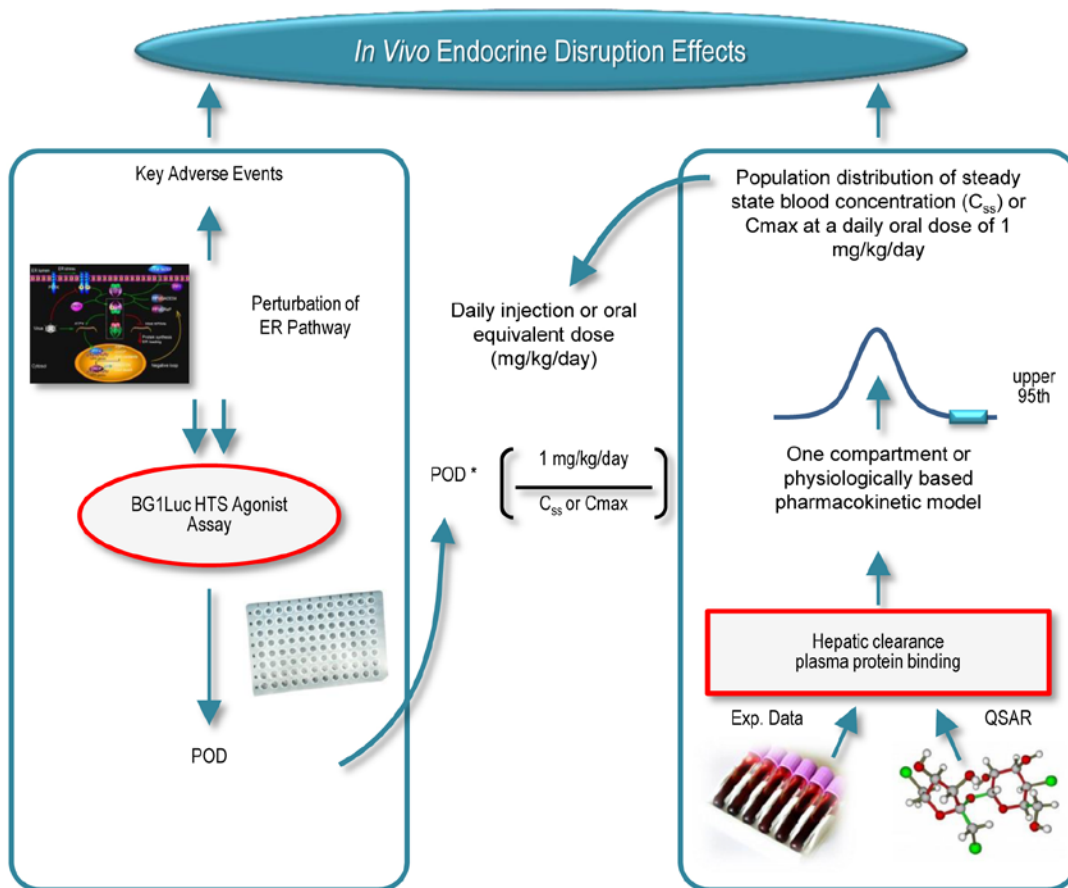
### Introduction

- 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).
- Efforts are ongoing within the U.S. federal Tox21 partnership to establish a testing strategy based on *in vitro* assays and *in silico* models that could speed up the screening process.

## Development of a Reverse Toxicokinetic Model for Estrogenic Effects

- The *in vitro* BG1Luc estrogen receptor (ER) transactivation assay (BG1Luc) is accepted internationally for identifying ER agonists and has been adapted to a high-throughput screening (HTS) format for use in Tox21 (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 and applied reverse pharmacokinetic modeling approaches for tested chemicals (**Figure 1**).
  - The point-of-departure (POD) is defined as the lowest nominal concentration in an *in vitro* assay that causes a response that significantly exceeds the background activity level. The PODs of three steroid estrogens commonly used as positive controls (17-beta estradiol, 17-alpha ethinylestradiol, and diethylstilbestrol) were derived from BG1Luc manual assays due to the limitation of tested concentration range in the BG1Luc HTS assays.
  - The one-compartment rat population pharmacokinetic (P-PK) model, built using the software package R (v. 3.1.2), assumes 100% absorption. This model was used to estimate median daily injection equivalent dose (IED) that would result in a steady-state blood concentration ( $C_{ss}$ ) equivalent to the POD in the BG1Luc HTS assay. The IED was then compared to the lowest “lowest effect level” (LEL) in the *in vivo* uterotrophic assay with an administration route of subcutaneous or intraperitoneal injection.
  - The one-compartment rat pharmacokinetic (GP-PK) model and rat physiologically based pharmacokinetic (GP-PBPK) model (**Figure 2**) were built using GastroPlus software (Simulations Plus, Inc.), which incorporates the Advanced Compartmental Absorption and Transit (ACAT) model consisting of nine compartments (stomach, duodenum, jejunum 1, jejunum 2, ileum 1, ileum 2, ileum 3, caecum, and ascending colon) to simulate GI tract absorption. Both GP models were used to estimate daily oral equivalent dose (OED) that would result in a maximum blood concentration ( $C_{max}$ ) equivalent to the POD in BG1Luc assay. The OED was then compared to the lowest LEL in the uterotrophic assay with oral administration route.

**Figure 1 Use of Pharmacokinetic Modeling for Reverse Dosimetry<sup>a</sup>**



Abbreviations: C<sub>max</sub> = maximum blood concentration; C<sub>ss</sub> = steady-state blood concentration; ER = estrogen receptor; Exp. = experimental; HTS = high-throughput screening; PK = pharmacokinetic; POD = point of departure; QSAR = quantitative structure–activity relationship.

<sup>a</sup> Adapted from Judson et al. 2011

### Data Used in the Analysis

- We selected 28 active ER reference chemicals for *in vitro* to *in vivo* extrapolation (IVIVE) analysis. The chemicals were selected according to collective results of high quality uterotrophic studies from literature reports (refer to **Ceger et al., SOT abstract 2641**, for more detailed discussion of literature review) and BG1Luc HTS assays. Of the 28 active ER reference chemicals, 27 chemicals had LELs from uterotrophic assays using injection routes of administration and 19 chemicals had oral dosing uterotrophic LELs.
- The fraction of unbound plasma protein (Fub) and intrinsic metabolic clearance rate (CL<sub>intrinsic</sub>) are the two most important parameters for model building. The values of Fub and CL<sub>intrinsic</sub> for these chemicals were obtained via a three-tiered strategy (**Table 1**).
  - If available, we used rat experimental values reported in the literature.
  - If rat experimental Fub values were not available, we used experimental Fub values determined with human plasma (Wetmore et al. 2012).
  - In most cases, the rat CL<sub>intrinsic</sub> values were calculated by scaling *in vitro* metabolic clearance (CL<sub>in vitro</sub>) determined using rat primary hepatocytes (Wetmore et al. 2013). If experimental measurements of rat CL<sub>in vitro</sub> were not available, CL<sub>in vitro</sub> determined using human primary hepatocytes was used to calculate rat CL<sub>intrinsic</sub> (Wetmore et al. 2012).
  - In cases where no experimental data were available for both species, predicted values from commercially available human QSAR models (ADMET Predictor™ [Simulations Plus, Inc.]) were applied. **Table 2** summarizes the performance of two human QSAR models used in this study when compared to experimental values from the rat. The ADMET Predictor plasma protein binding model directly predicts Fub based on chemical structure. The enzymatic clearance models predict unbound *in vitro* microsomal clearance for each cytochrome P450 enzyme identified as the source of clearance for a chemical. The sum of microsomal clearance was then converted to CL<sub>intrinsic</sub> after incorporating rat liver physiology.

**Table 1 PK Parameters Used in the Models**

Chemical	Fub	CLintrinsic (L/h)	Source_Fub	Source_CLintrinsic
Fenarimol	0.028	0.000	Rat Exp <sup>a</sup>	Rat Exp <sup>a</sup>
17beta-Estradiol	0.053	1.000	Rat Exp <sup>b</sup>	Rat Exp <sup>b</sup>
Bisphenol A	0.06	0.155	Rat Exp <sup>b</sup>	Rat Exp <sup>a</sup>
Genistein	0.3	1.246	Rat Exp <sup>c</sup>	Hum Exp <sup>d</sup>
17alpha-Ethinyl estradiol	0.47	1.483	Rat Exp <sup>f</sup>	QSAR
4-tert-Octylphenol	0.019025	1.799	Hum Exp <sup>g</sup>	Hum Exp <sup>d</sup>
Diethylstilbestrol	0.005	2.753	Hum Exp <sup>g</sup>	Hum Exp <sup>d</sup>
Bisphenol B	0.01823	2.378	Hum Exp <sup>g</sup>	Hum Exp <sup>d</sup>
Methoxychlor	0.005	1.957	Hum Exp <sup>d</sup>	Hum Exp <sup>d</sup>
o,p'-DDT	0.005	1.006	Hum Exp <sup>d</sup>	Hum Exp <sup>d</sup>
4-(1,1-Dimethylpropyl)phenol	0.005	1.817	Hum Exp <sup>d</sup>	Hum Exp <sup>d</sup>
Butylparaben	0.041572	2.621	Hum Exp <sup>d</sup>	Hum Exp <sup>d</sup>
17alpha-Estradiol	0.02	0.401	Hum Exp <sup>h</sup>	QSAR
Norethindrone	0.2	0.695	Hum Exp <sup>h</sup>	QSAR
Mestranol	0.02	1.003	Hum Exp <sup>h</sup>	QSAR
Estrone	0.0371	0.354	Hum Exp <sup>i</sup>	QSAR
4-Dodecylphenol	0.01	4.171	QSAR	QSAR
Benzophenone-2	0.0371	0.229	QSAR	QSAR
2,4-Dihydroxybenzophenone	0.0284	1.888	QSAR	QSAR
Bisphenol AF	0.011	155.940	QSAR	QSAR
Zearalenone	0.0414	0.276	QSAR	QSAR
Equilin	0.0548	1.214	QSAR	QSAR
Estriol	0.0861	0.000	QSAR	QSAR
Benzoic acid, 4-hydroxy-, 2-ethylhexyl ester	0.0231	1.270	QSAR	QSAR
5alpha-Dihydrotestosterone	0.0849	0.804	QSAR	QSAR
17alpha-Methyltestosterone	0.0673	0.751	QSAR	QSAR
4-Cumylphenol	0.0319	2.624	QSAR	QSAR
Bisphenol S	0.1323	0.138	QSAR	QSAR

Abbreviations: CLintrinsic = intrinsic metabolic clearance rate; Fub = fraction of chemical unbound in the plasma; PK = pharmacokinetic; QSAR = human value predicted from quantitative structure–activity relationship software. Rat\_Exp and Hum\_Exp refer to rat or human experimental data reported from literature. a. Wetmore et al. 2013; b. Plowchalk and Teeguarden 2002; c. Lu et al. 1998; d. Wetmore et al. 2012; e. Schlosser et al. 2006; f. Grabowski et al. 1984; g. Wetmore et al. unpublished data; h. Zhu et al. 2013; i. Speight et al. 1979.

**Table 2 Performance Evaluation of QSAR Model Prediction on Rat PK Parameters**

Comparison (n=57)	Correlation Coefficient	MAE	RMSE	MSR
Rat Fub (%) Exp. vs. Hum. Fub (%) Exp.	0.64	9.68	20.28	0.54
Rat Fub (%) Exp. vs. Hum. Fub QSAR Model Prediction	0.77	8.96	15.95	0.68
Rat CLintrinsic Exp. vs. Hum. CLintrinsic Exp. Scaled to Rat	0.61	0.69	1.10	0.81
Rat CLintrinsic Exp. vs. QSAR Prediction Using Hum. <i>In Vitro</i> Microsome Clearance Model	0.31	2.25	3.31	0.92

Abbreviations: CLintrinsic = intrinsic metabolic clearance rate; Exp = experimental value; Fub = fraction of chemical unbound in the plasma; Hum. = human; MAE = mean absolute error; MSR = mean standardized residuals; RMSE = root mean square error; QSAR = quantitative structure–activity relationship.

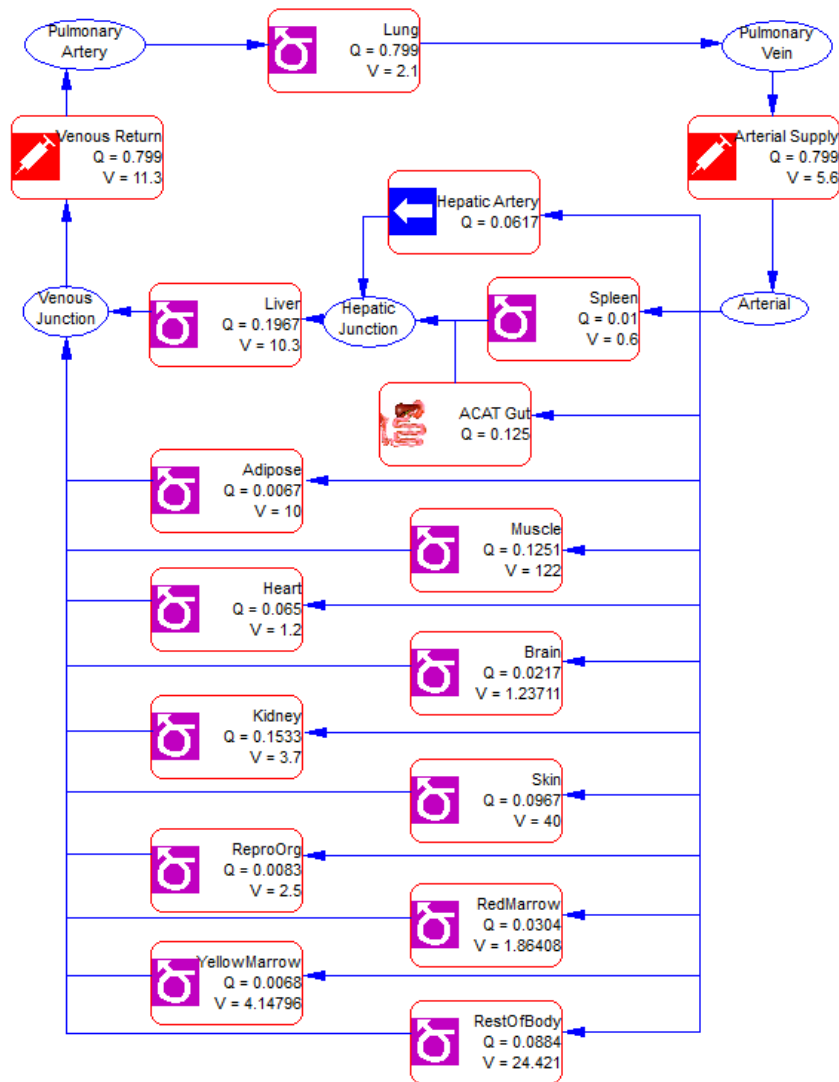
- For all three models (P-PK, GP-PK, and GP-PBPK), the hepatic clearance (CL<sub>hepatic</sub>) and renal clearance (CL<sub>renal</sub>) were calculated using the following equations:

$$CL_{hepatic} (L/h) = Q_{liver} (L/h) * \frac{Fub * CL_{intrinsic}}{Q_{liver} + Fub * CL_{intrinsic}}$$

$$CL_{renal} (L/h) = GFR(L/h) * Fub$$

- In these equations, GFR is glomerular filtration rate and Q<sub>liver</sub> is liver blood flow rate.
- For GP-PBPK, the tissue partition coefficients for each chemical were predicted using ADMET Predictor.
- For one chemical, bisphenol A, there was a published PBPK model (Yang et al. 2013) for oral administration based on experimentally measured time course data.

**Figure 2 Structure of the GastroPlus Rat PBPK Model**



Abbreviations: ACAT = advanced compartmental absorption and transit model; PBPK = physiologically based pharmacokinetic; Q = blood flow; V = volume.

## Results

- The ADMET Predictor human Fub model was able to predict rat Fub with a correlation coefficient of 0.77 and mean absolute error less than 10 in terms of percentage of plasma protein binding. The enzymatic clearance models did not perform as well as the Fub model, but a weak correlation between the model prediction and experimental values for rat CLintrinsic is clearly shown.
- The median IEDs estimated by the P-PK model were lower than the lowest LELs in uterotrophic injection studies for 26 of 27 active ER reference chemicals (**Table 3, Figure 3**).
  - The IED estimates for 10 of the 27 chemicals were within 20-fold of the lowest LELs in uterotrophic injection studies, among which 6 chemicals used human QSAR prediction values of Fub and/or CLintrinsic.
- The median OEDs estimated by the GP-PK and GP-PBPK models were lower than the lowest LELs in uterotrophic oral studies assays for all 19 active ER reference chemicals (**Table 4, Figure 4**).
  - The OED estimates for 3 of the 19 chemicals were within 20-fold of the lowest LELs in uterotrophic oral studies, among which one chemical (methoxychlor) used human experimental Fub and CLintrinsic values and another chemical (mestranol) used human experimental Fub value and QSAR prediction for CLintrinsic.



**Table 3 Median IEDs Estimated from PODs of *In Vitro* Assay by P-PK Model Compared to Lowest Injection LELs in Uterotrophic Assays**

Chemical	L_LEL from UT assay_Injection (mg/kg/day) <sup>a</sup>	POD (μM) from BG1Luc HTS Assay	Median IED (mg/kg/day)	Ratio: L_LEL/IED <sup>b</sup>
17beta-Estradiol	1.00E-04	3.18E-06 <sup>c</sup>	4.45E-06	22.46
17alpha-Ethinyl estradiol	1.00E-04	2.93E-06 <sup>c</sup>	3.41E-05	2.93
Diethylstilbestrol	2.50E-04	1.56E-05 <sup>c</sup>	5.53E-06	45.23
Mestranol	1.60E-03	0.001	5.99E-04	2.67
Estrone	2.00E-03	0.002	9.27E-04	2.16
17alpha-Estradiol	5.00E-03	0.001	2.94E-04	17.02
Estriol	0.04	0.001	1.58E-04	253.56
<b>Methoxychlor</b>	<b>0.75</b>	<b>4.685</b>	<b>1.549</b>	<b>0.48</b>
Genistein	1	0.049	0.355	2.81
o,p'-DDT	1	0.630	0.114	8.76
Bisphenol A	2	0.166	0.050	39.87
Norethindrone	2	0.017	0.064	31.03
Zearalenone	2	0.001	3.52E-04	5686.94
Equilin	2	0.001	1.99E-03	1004.74
Bisphenol AF	4	0.030	0.527	7.58
5alpha-Dihydrotestosterone	4	0.040	0.076	52.31
17alpha-Methyltestosterone	10	0.023	0.035	288.38
Bisphenol B	20	0.071	0.070	287.73
4-Cumylphenol	20	0.290	0.459	43.55
Bisphenol S	20	1.157	0.786	25.44
4-Dodecylphenol	40	0.316	0.320	125.19
Butylparaben	70	3.023	5.556	12.60
2,4-Dihydroxybenzophenone	100	5.085	5.435	18.40
4-tert-Octylphenol	200	0.627	0.423	472.75
4-(1,1-Dimethylpropyl)phenol	200	20.876	3.070	65.15
Benzophenone-2	200	0.995	0.265	755.77
Benzoic acid, 4-hydroxy-, 2-ethylhexyl ester	200	0.846	0.608	329.08

Abbreviations: ER = estrogen receptor; HTS = high-throughput screening; IED = daily injection equivalent dose; LEL = lowest effective level; L\_LEL = lowest LEL; PK = pharmacokinetic; POD = point of departure; UT = uterotrophic.

a The table is sorted by L\_LEL from UT assay\_Injection (mg/kg/day) in ascending order.

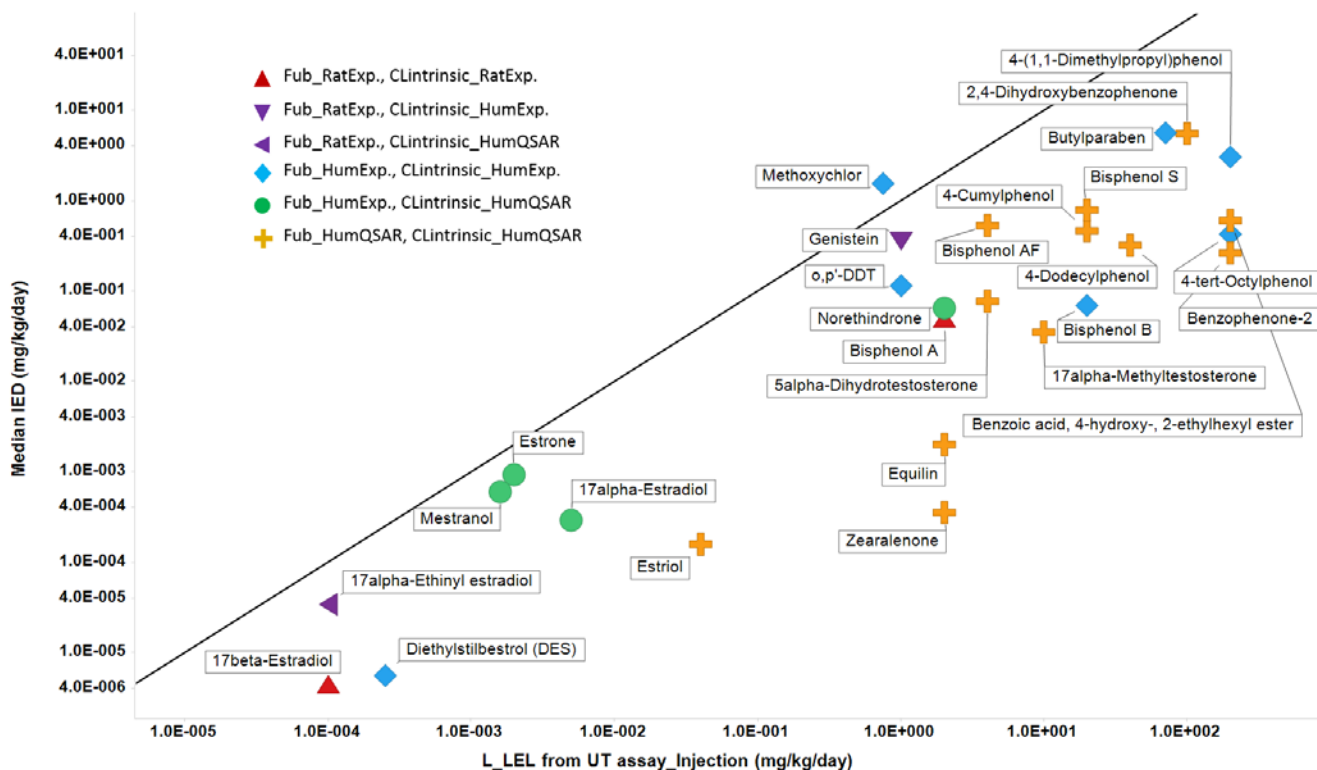
b Rows with text in **boldface** indicate IED estimates that are larger than the L\_LEL. Shaded rows indicate IED estimates within 20-fold of the L\_LEL in uterotrophic assays.

c The POD values were derived from BG1Luc manual assays.

- **Figure 3** is a graphical representation of the data in **Table 3**. The horizontal axis represents the log value of lowest LEL (mg/kg/day) from uterotrophic injection studies. The vertical axis represents the log value of median IED estimated using the P-PK model that result in a  $C_{ss}$  equivalent to POD in the BG1Luc HTS assay.

Figure 3

Estimated Median IEDs from POD Using  $C_{ss}$  and Lowest Injection LELs in Uterotrophic Assays<sup>a</sup>



Abbreviations: CLintrinsic = intrinsic metabolic clearance rate; Exp. = experimental; Fub = fraction of chemical unbound in the plasma; Hum = human; IED = daily injection equivalent dose; LEL = lowest effect level; LEL = lowest LEL; QSAR = quantitative structure–activity relationship; UT = uterotrophic.

a The black line represents  $y = x$ . The symbols represent different sources of Fub and CLintrinsic used in the P-PK model; refer to **Table 1** for details.

**Table 4 OEDs Estimated from PODs of *In Vitro* Assay Compared to Lowest Oral LEL of Uterotrophic Assays**

Chemical	L_LEL from UT assay_Oral (mg/kg/day) <sup>a</sup>	POD (µM) from BG1Luc HTS Assay	OED (mg/kg/day) (GP-PK model)	OED (mg/kg/day) (GP-PBPK model)	Ratio: L_LEL/OED (GP-PBPK model)
17alpha-Ethinyl estradiol	2.00E-04	2.93E-06 <sup>b</sup>	6.63E-06	5.96E-06	33.6
Diethylstilbestrol (DES)	1.00E-03	1.56E-05 <sup>b</sup>	4.52E-06	4.10E-06	243.9
Mestranol	2.76E-03	0.001	4.30E-04	3.18E-04	8.7
17beta-Estradiol	5.00E-03	3.18E-06 <sup>b</sup>	1.80E-06	1.66E-06	3010.5
Estrone	0.02	0.002	6.28E-04	5.83E-04	33.9
Estriol	0.03	0.001	1.33E-04	1.04E-04	325.6
17alpha-Estradiol	0.4	0.001	2.43E-04	2.13E-04	1882.2
Norethindrone	0.5	0.017	0.013	0.011	45.9
Zearalenone	8	0.001	2.20E-04	2.03E-04	39486.7
o,p'-DDT	10	0.630	0.149	0.222	45.0
17alpha-Methyltestosterone	15	0.023	0.014	0.012	1279.0
Genistein	20	0.049	0.030	0.039	513.3
Methoxychlor	20	4.685	1.347	1.683	11.9
4-tert-Octylphenol	56	0.627	0.273	0.703	79.6
Bisphenol A	200	0.166	68.66 <sup>c</sup>	68.66 <sup>c</sup>	2.9
Fenarimol	200	16.264	1.801	1.362	146.9
Butylparaben	400	3.023	0.891	0.972	411.4
Benzophenone-2	1000	0.995	0.152	0.088	11363.6
2,4-Dihydroxybenzophenone	1000	5.085	1.560	1.055	948.0

Abbreviations: ER = estrogen receptor; GP = GastroPlus; HTS = high-throughput screening; LEL = lowest effective level; L\_LEL = lowest LEL; OED = daily oral equivalent dose; PBPK = physiologically based pharmacokinetic; PK = pharmacokinetic; POD = point of departure; UT = uterotrophic.

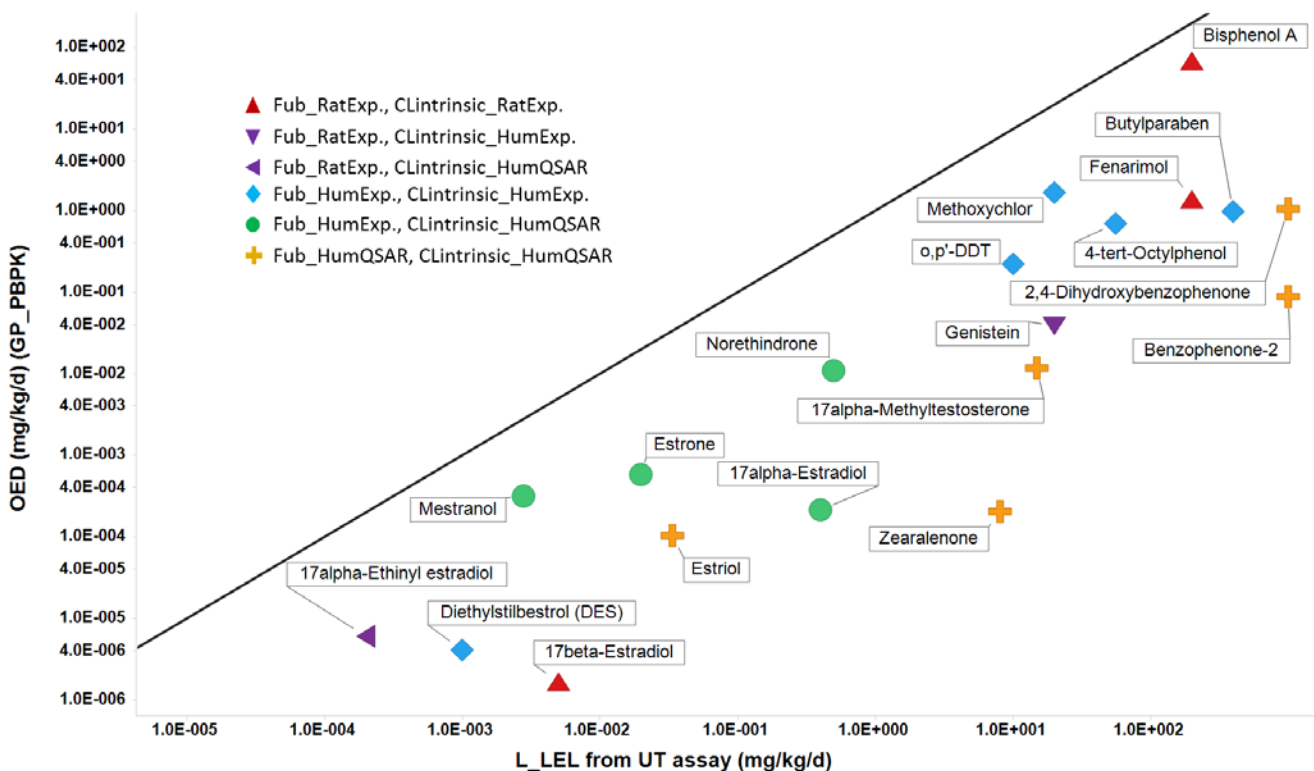
a Table is sorted by L\_LEL from UT assay\_Oral (mg/kg/day) in ascending order.

b The POD values were derived from BG1Luc manual assays.

c OED for bisphenol A was estimated from the published PBPK model (Yang et al. 2013).

- **Figure 4** is a graphical representation of the data in **Table 4**. The horizontal axis represents the log value of lowest LEL (mg/kg/day) from the oral uterotrophic assays. The vertical axis represents the log value of OED estimated using GastroPlus rat PBPK (GP-PBPK) model that results in a Cmax equivalent to the POD in the BG1Luc HTS assay.

**Figure 4. Estimated OEDs from PODs Using Cmax and Lowest Oral LELs of Uterotrophic Assays<sup>a</sup>**



Abbreviations: CLintrinsic = intrinsic metabolic clearance rate; Cmax = maximum blood concentration; Exp. = experimental; Fub = fraction of chemical unbound in the plasma; GP\_PBPBK = GastroPlus rat physiologically based pharmacokinetic; Hum = human; LEL = lowest effective level; L\_LEL = lowest LEL; OED = daily oral equivalent dose; POD = point of departure; QSAR = quantitative structure–activity relationship; UT = uterotrophic.

<sup>a</sup> The black line represents  $y = x$ . The symbols represent different sources of Fub and CLintrinsic used in the GP-PBPBK model; refer to **Table 1** for details.

## Discussion and Conclusion

- The high concordance between *in vitro* and *in vivo* endpoints supports the use of the BG1Luc HTS assay as a screen for potential endocrine-disrupting chemicals.
- The applicability of IVIVE can be improved significantly by using validated and more complex PK and PBPK models.
- For almost all the tested chemicals, the IEDs and OEDs estimated from the POD of BG1Luc HTS assay are smaller than the lowest LELs in corresponding uterotrophic assays, suggesting the *in vitro* data provide a more conservative hazard estimate (Tables 3 and 4).
- About 16%-40% of chemicals have IEDs or OED estimates within 20-fold of the lowest LELs in uterotrophic studies.
  - This suggests that our IVIVE approach works for a subset of chemicals including a few chemicals with Fub and CLintrinsic values predicted from human QSAR models, which sheds light on further effort in quantitatively predicting *in vivo* effects and for proper interpretation of *in vitro* data for risk assessments.
  - The IEDs or OED estimates for some chemicals were 3-4 order of magnitude lower than the lowest LELs in uterotrophic studies, which will need further investigation.
- The nominal effective concentration in the *in vitro* assay should be adjusted for important toxicokinetic factors to more accurately predict *in vivo* effects.

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