In Vitro to In Vivo Extrapolation for Estrogenic Activity of Environmental Chemicals

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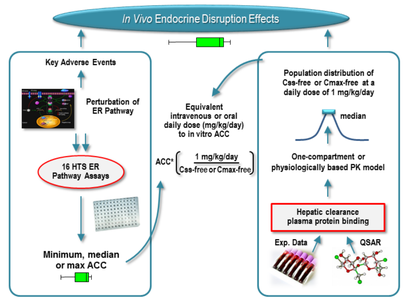
Introduction

- In vitro high throughput screening (HTS) assays can accelerate and reduce the overall cost of identifying potentially toxic chemicals. These assays are being developed and evaluated in programs such as the U.S. federal Tox21 consortium (Tice et al. 2013) and the U.S. Environmental Protection Agency's ToxCast project (Kavlock et al. 2012).
- However, before in vitro HTS assays can be used in risk assessment, the ability of in vitro activity to predict in vivo dose-response relationships needs to be evaluated. Central to this evaluation is determining how closely a bioactive chemical concentration in an in vitro assay corresponds to the blood and tissue levels of that chemical that cause adverse effects in vivo.
- Generally, an in vitro concentration-activity relationship is described using nominal tested concentration, the amount of chemical added in the medium divided by volume of the exposure medium. However, whether the nominal concentration approximates total or free chemical concentration is context-dependent and often not evaluated. In a serum-free medium, one would assume that the nominal concentration approximates free chemical concentration, instead of total chemical concentration.
- In this study, we evaluated the performance of pharmacokinetic (PK) and physiologically based (PBPK) models for in vitro to in vivo extrapolation (IVIVE) of estrogen receptor (ER) pathway activity, assuming that nominal in vitro HTS concentrations approximate free chemical concentrations, which are expected to be bioactive.

Data Used in the Analysis

- We selected 32 ER-active chemicals according to availability of data from in vitro HTS assays and data from high-quality in vivo uterotrophic (UT) assay studies (**Table 1**).
- In vitro data were obtained for these chemicals from 16 ToxCast/Tox21 HTS assays that measure many key events along the ER pathway (e.g. receptor binding, transcription, and cell proliferation) (Judson et al. 2015).
  - For each chemical-assay pair, we calculated the activity concentration at cutoff (ACC) as the lowest effective concentration (LEC) that causes a statistically significant estrogenic effect in the assay.
  - We calculated the minimum, median, and maximum ACC for all 16 assays (**Table 1**).
  - In some cases, maximum responses were observed at the lowest tested concentration. In those cases, an EC10 value (concentration at which 10% of maximum activity is observed) from the manual BG1Luc ER TA assay was used to replace the minimum ACC for that chemical. This was because all chemicals were tested in the BG1Luc ER TA manual assay at concentrations low enough to produce no response (Ceger et al. 2015).
- The UT assay measures uterine hypertrophy caused by activation of the ER pathway (Kleinstreuer et al. 2015).
  - For each chemical-UT assay pair, we derived the lowest effective dose level (LEL) that caused an estrogenic effect. We calculated the lowest, median, and maximum LEL for each chemical to cover the range of in vivo responses.
  - Data to derive the LEL were available for subcutaneous or intraperitoneal injection for all 32 chemicals and for oral administration for 18 out of 32 chemicals.
- Values for the fraction of chemical unbound to plasma proteins (Fub) and intrinsic metabolic clearance rate (CLintrinsic), two parameters used for model building, were obtained as follows (**Table 2**):
  - If available, we used rat Fub experimental values reported in the literature.
    - When rat experimental data were not available, we used human Fub experimental values instead (Wetmore et al. 2012). The correlation coefficient between rat and human experimental Fub measurements is shown in **Table 3.**
    - If no experimental data were available for either species, we used predicted values from commercially available human quantitative structure-activity relationship (QSAR) models (ADMET Predictor™ [Simulations Plus, Inc.]). The performance of QSAR model prediction in terms of mean absolute error (MAE) and root mean square error (RMSE) is summarized in **Table 3**.
  - If available, rat CLintrinsic values were calculated by scaling to the whole liver from in vitro metabolic clearance rate (CLinvitro) values determined using rat primary hepatocytes (Wetmore et al. 2013).
    - When experimental measurements of rat CLinvitro were not available, CLinvitro values determined using human primary hepatocytes were used to calculate rat CLintrinsic values (Wetmore et al. 2012). The correlation coefficient between rat and human experimental CLinvitro values is shown in **Table 3.**
    - If no experimental data were available, values predicted from a quantitative property-property relationship (QPPR) model were used (Kirman et al. 2015). The QPPR model predicts CLintrinsic using octanol-water and water-air partition coefficients that can be quickly estimated for most chemicals. The performance of QPPR model prediction in terms of MAE and RMSE are summarized in **Table 3.**

Figure 1. A Reverse Pharmacokinetic Model for In Vitro to In Vivo Extrapolation[a]



Abbreviations: ACC = activity concentration at cutoff; Cmax = maximum blood concentration; Cmax-free = estimate of maximum concentration of free chemical in the blood; Css = steady-state blood concentration; Css-free = estimate of steady-state concentration of free chemical in the blood; ER = estrogen receptor; Exp. = experimental; HTS = high-throughput screening; PK = pharmacokinetic; QSAR = quantitative structure-activity relationship.

[a] Figure adapted from Judson et al. 2011

Table 1. ACC Values for Chemicals Used in the Analysis

Chemical	CASRN	Minimum ACC (uM)[b]	Median ACC(uM)	Maximum ACC(uM)
17beta-Estradiol	50-28-2	1.080E-06[b]	0.001	0.534
Equilin	474-86-2	1.71E-07	0.002	0.053
17alpha-Estradiol	57-91-0	1.69E-06	7.37E-04	0.009
Ethinyl estradiol	57-63-6	1.389E-06[b]	3.54E-04	0.009
Diethylstilbestrol	56-53-1	7.361E-06[b]	4.72E-04	0.008
Mestranol	72-33-3	5.37E-06	0.026	1.533
Clomiphene citrate	50-41-9	1.02E-05	3.25E-04	0.066
Tamoxifen	10540-29-1	9.806E-04[b]	0.027	76.001
Estrone	53-16-7	2.825E-05[b]	0.004	0.036
Estriol	50-27-1	2.01E-05	9.86E-04	0.191
Norethindrone	68-22-4	8.01E-05	0.418	10.650
Zearalenone	17924-92-4	9.90E-05	0.004	0.091
Methyltestosterone	58-18-4	2.23E-04	1.905	17.325
Genistein (4',5,7-trihydroxyisoflavone)	446-72-0	0.004	0.085	7.406
4,4'-(Hexafluoroisopropylidene) diphenol	1478-61-1	0.011	0.030	0.572
2,2',4,4'-Tetrahydroxybenzophenone	131-55-5	0.014	0.845	11.137
Dihydrotestosterone	521-18-6	0.015	2.092	18.801
o,p'-DDT	789-02-6	0.016	0.701	3.120
p-Dodecyl-phenol	104-43-8	0.023	0.631	52.104
Bisphenol B	77-40-7	0.032	0.100	0.306
Bisphenol A	80-05-7	0.033	0.185	1.388
Nonylphenol (mixture of branched chains)	25154-52-3	0.039	0.600	7.614
4-Tert-octylphenol	140-66-9	0.075	0.774	5.965
p-Cumylphenol	599-64-4	0.087	0.684	5.012
2-Ethylhexyl-p-hydroxybenzoate	5153-25-3	0.151	1.025	27.782
p-(Tert-pentyl)phenol	80-46-6	0.280	1.834	43.951
4,4'-Sulfonyldiphenol	80-09-1	0.566	11.582	38.533
2,4-Dihydroxybenzophenone	131-56-6	0.594	1.975	23.692
Methoxychlor	72-43-5	0.676	2.348	5.530
Butylparaben	94-26-8	0.736	1.945	20.755
p-t-Butylphenol	98-54-4	0.858	9.664	33.621
Nonylphenol	104-40-5	1.461	7.768	15.472

Abbreviations: ACC = activity concentration at cutoff; CASRN = Chemical Abstracts Service Registry Number.

[a] The table is sorted by minimum ACC in ascending order.

[b] An EC10 value from manual BG1Luc ER TA assay was used to replace the minimum ACC as described in the preceding text.

Table 2. PK Parameters Used in the Models

Chemical	Fub	CLintrinsic (L/h)	Renal Clearance (L/h)	Source of PK Parameters (Fub, CLintrinsic)
17beta-Estradiol	0.053	1	0.0042	Rat_Exp[a], Rat_Exp[a]
Bisphenol A	0.06	0.155	0.0048	Rat_Exp[b] Rat_Exp[c]
Genistein (4',5,7-trihydroxyisoflavone)	0.3	1.246	0.024	Rat_Exp[a], Hum_Exp[a]
Ethinyl estradiol	0.47	1.603	0.0376	Rat_Exp[c], QPPR
Methoxychlor	0.005	11.363	0.0004	Hum_Exp[d], Hum_Exp[d]
Diethylstilbestrol	0.005	2.753	0.0004	Hum_Exp[d], Hum_Exp[d]
Tamoxifen	0.005	0.568	0.0004	Hum_Exp[d], Hum_Exp[d]
o,p'-DDT	0.005	1.006	0.0004	Hum_Exp[d] Hum_Exp[d]
Bisphenol B	0.018	2.378	0.0015	Hum_Exp[b] Hum_Exp[c]
4-Tert-ctylphenol	0.019	1.799	0.0015	Hum_Exp[b] Hum_Exp[c]
p-(Tert-pentyl)phenol	0.005	1.398	0.0004	Hum_Exp[d], Hum_Exp[d]
Butylparaben	0.042	2.621	0.0033	Hum_Exp[d], Hum_Exp[d]
p-t-butylphenol	0.105	1.288	0.0084	Hum_Exp[b] Hum_Exp[c]
Estrone	0.037	1.838	0.003	Hum_Exp[c], QPPR
Nonylphenol (mixture of branched chains)	0.019	1.46	0.0015	QSAR, Hum_Exp[c]
Equilin	0.055	1.728	0.0044	QSAR, QPPR
17alpha-estradiol	0.042	1.763	0.0033	QSAR, QPPR
Mestranol	0.031	1.546	0.0024	QSAR, QPPR
Clomiphene citrate	0.015	0.591	0.0012	QSAR, QPPR
Estriol	0.086	1.98	0.0069	QSAR, QPPR
Norethindrone	0.128	2.11	0.0103	QSAR, QPPR
Zearalenone	0.041	1.482	0.0033	QSAR, QPPR
Methyltestosterone	0.067	2.135	0.0054	QSAR, QPPR
4,4'-(Hexafluoroisopropylidene) diphenol	0.011	1.876	0.0009	QSAR, QPPR
2,2',4,4'-Tetrahydroxybenzophenone	0.037	1.207	0.003	QSAR, QPPR
Dihydrotestosterone	0.085	2.408	0.0068	QSAR, QPPR
p-Dodecyl-phenol	0.01	0.603	0.0008	QSAR, QPPR
p-Cumylphenol	0.032	1.948	0.0026	QSAR, QPPR
2-Ethylhexyl-p-hydroxybenzoate	0.023	1.258	0.0018	QSAR, QPPR
4,4'-Sulfonyldiphenol	0.132	1.005	0.0106	QSAR, QPPR
2,4-Dihydroxybenzophenone	0.028	1.675	0.0023	QSAR, QPPR
Nonylphenol	0.017	1.047	0.0014	QSAR, QPPR

Abbreviations: CLintrinsic = intrinsic metabolic clearance rate; Fub = fraction of chemical unbound in the plasma; Hum_Exp = human experimental data reported from literature; PK = pharmacokinetic; QPPR = value predicted from quantitative property-property relationship; QSAR = human value predicted from quantitative structure-activity relationship software; Rat_Exp = rat experimental data reported from literature.

Literature sources for PK parameters are as follows: (a) Plowchalk et al. 2002; (b) Teeguarden et al. 2005; (c) Wetmore et al. 2013; (d) Wetmore et al. 2012; (e) Schlosser et al. 2006; (f) Grabowski et al. 1984; (g) Wetmore et al. unpublished data; (h) Speight et al. 1979.

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Table 3. Correlation between Human and Rat Experimental Values and Performance of Model Predictions of PK Parameters

Comparison		Model Validation Parameter			Experimental Value Range
		Correlation Coefficient	MAE	RMSE	
Fub	Human Exp. vs Rat Exp. (n=57)	0.64	0.097	0.2	0 – 1
	QSAR vs Rat Exp. (n=66)		0.11	0.2	0 – 1
	QSAR vs Human Exp. (n=419)		0.1	0.18	0 – 1
CLintrinsic (L/h)	Human Exp.(Scaled to Rat) vs Rat Exp. (n=57)	0.61	0.69	1.1	0 – 4.88 (L/h)
	QPPR vs Rat Exp. (n=57)		1.69	2.61	0 – 4.88 (L/h)
	QPPR vs Human Exp. (n=439)		153.67	346.3	0 – 4297.7 (L/h)

Abbreviations: CLintrinsic = intrinsic metabolic clearance rate; Exp = experimental value; Fub = fraction of chemical unbound in the plasma; MAE = mean absolute error; PK = pharmacokinetic; RMSE = root mean square error; QPPR = value predicted from quantitative property-property relationship (Kirman et al. 2015); QSAR = quantitative structure-activity relationship.

Development of an IVIVE Model for Estrogenic Effects

- We applied either a one-compartment rat population pharmacokinetic (P-PK) or rat oral multi-compartment physiologically based PK (O-PBPK) model to estimate the daily equivalent administered dose (EAD) that would result in free chemical concentrations in the blood equivalent to the lowest, median, or maximum ACC value across assays.
  - The P-PK model was built using the software package R (version 3.1.2; R Core Team 2013) and used to estimate daily EAD_Inj (Inj: injection) after administration of chemical by injection.
    - The model estimates the steady-state blood concentration (Css) assuming 100% absorption (Wetmore et al. 2013).
    - The term EAD_Inj refers to the equivalent administered dose (EAD) that could result in free blood chemical concentrations (Css-free) equivalent to corresponding ACCs through injection route. Css-free is calculated as Css times Fub.
  - The O-PBPK model was built using GastroPlus software (Simulations Plus, Inc.) and used to simulate the daily EAD_Oral after ingestion of chemical.
    - The model incorporates the advanced compartmental absorption and transit (ACAT) model to simulate chemical absorption through the gastrointestinal tract and estimates the maximum blood concentration (Cmax) (**Figure 2**).
    - The chemical tissue partition coefficients used in the model were predicted using ADMET Predictor (Simulations Plus, Inc.).
    - The term EAD_Oral refers to the EAD that could result in free blood concentrations (Cmax-free) equivalent to corresponding ACCs through oral route. Cmax-free is calculated as Cmax times Fub.
  - For both models, hepatic clearance (CLhepatic) and renal clearance (CLrenal) were calculated using the following equations:

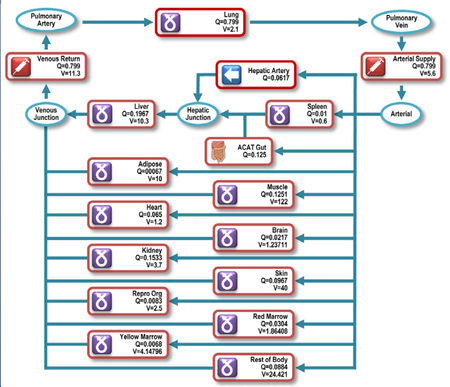
$$CLhepatic\ (L/h) = Qliver\ (L/h) * \frac{Fub * CLintrinsic}{Qliver + Fub * CLintrinsic}$$

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

In these equations, GFR is glomerular filtration rate and Qliver is liver blood flow rate. The renal clearance refers to non-metabolic clearance only.

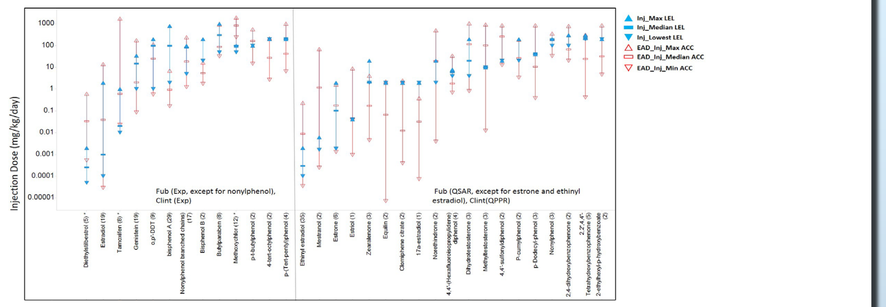
- For bisphenol A, we also evaluated a published PBPK model that incorporates glucuronidation (Yang et al. 2013) (**Figure 4**).
- The range of EAD_Inj and EAD_Oral were compared to the range of LELs from UT assays with corresponding administration routes, respectively (**Figures 3** and **4**).

Figure 2. Structure of the GastroPlus Rat PBPK Model



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

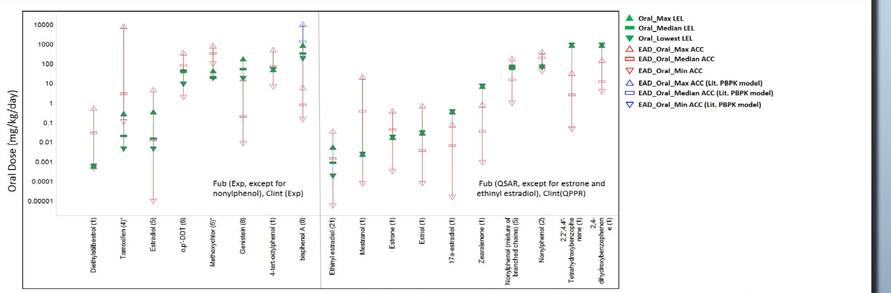
Figure 3. Range of EAD_Inj Estimated from P-PK Model Compared to UT Injection LELs



Abbreviations: ACC = activity concentration at cutoff; Clint = intrinsic metabolic clearance rate; EAD_Inj = daily injection equivalent dose that results in free chemical concentrations in the blood equivalent to corresponding ACCs; Exp = experimental; Fub = fraction of chemical unbound in the plasma; Inj. = injection; LEL = lowest effect level; PK = pharmacokinetic; P-PK = one-compartment population pharmacokinetic; UT = uterotrophic.

Numbers in parentheses indicate the number of guideline-like UT injection studies (Kleinstreuer et al. 2015). Asterisks indicate cases in which the EAD_Inj overestimated the injection effective dose.

Figure 4. Range of EAD_Oral Estimated from O-PBPK Model Compared to UT Oral LELs



Abbreviations: ACC = activity concentration at cutoff; Clint = intrinsic metabolic clearance rate; Exp = experimental; Fub = fraction of chemical unbound in the plasma; EAD_Oral = daily oral equivalent dose that results in free chemical concentrations in the blood equivalent to corresponding ACCs; LEL = lowest effect level; Lit. = literature; O-PBPK = oral physiologically based pharmacokinetic; PK = pharmacokinetic; QPPR= quantitative property-property relationship; QSAR = quantitative structure-activity relationship; UT = uterotrophic.

Numbers in parentheses indicate the number of guideline-like UT injection studies (Kleinstreuer et al. 2015). Asterisks indicate cases in which the EAD_Oral overestimated the oral effective dose. Liturature PBPK model is from Yang et al. 2013.

Results

- **Table 3** summarizes the results of analyses of (1) the correlation of experimental measurements of the two PK parameters between species and (2) the performance of the QSAR and QPPR models in estimating PK parameters.
- There was a positive correlation between human and rat experimental values of Fub and CLintrinsic. The human QSAR model predicted rat Fub well with an MAE of 0.11 corresponding to an experimental value range of 0-1. The QPPR model also predicted CLintrinsic well with an MAE of 1.69 corresponding to an experimental value range of 0-4.88 (L/h).
- The range of EAD_Inj estimated by the P-PK model covered the range of LELs in injection UT studies for 25 of 32 chemicals (**Figure 3**). The EAD_Inj underpredicted injection LELs for four chemicals (17alpha-estradiol, bisphenol A, bisphenol B, and zearalenone) and overpredicted injection LELs for three chemicals (DES, methoxychlor, and tamoxifen).
- The range of EAD_Oral estimated by the O-PBPK model covered the range of LELs in oral UT studies for 11 of 18 chemicals (**Figure 4**). The EAD_Oral underpredicted oral LELs for six chemicals (2,2',4,4'-tetrahydroxybenzophenone, 2,4-dihydroxybenzophenone, 17alpha-estradiol, bisphenol A, genistein, and zearalenone), and overpredicted oral LELs for two chemicals (methoxychlor and tamoxifen).
- For both injection and oral studies, the use of experimental or predicted values of Fub and/or CLintrinsic had no significant impact on predicting LELs.

Discussion and Conclusion

- The range of EAD-free estimates correlated well with the range of in vivo UT LELs for the majority of chemicals tested for both oral and injection administration routes. This suggests that this IVIVE approach could provide valid estimates of in vivo doses.
- In cases where no experimental measurements were available, the current QSAR and QPPR models provided an effective way to estimate PK parameters for IVIVE analysis.
- The metabolic clearance that we incorporated in our models are mainly hepatic clearance. Consideration of metabolic activity due to gut metabolism or extrahepatic metabolism could further improve accuracy of the IVIVE approach. For example:
  - The P-PK model underpredicted LELs in injection UT studies for bisphenol A and bisphenol B. These which could be improved by incorporating glucuronidation in gut absorption.
  - The O-PBPK overpredicted LELs in UT studies for methoxychlor, an error that could be due to lack of metabolism in the in vitro assays.

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