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

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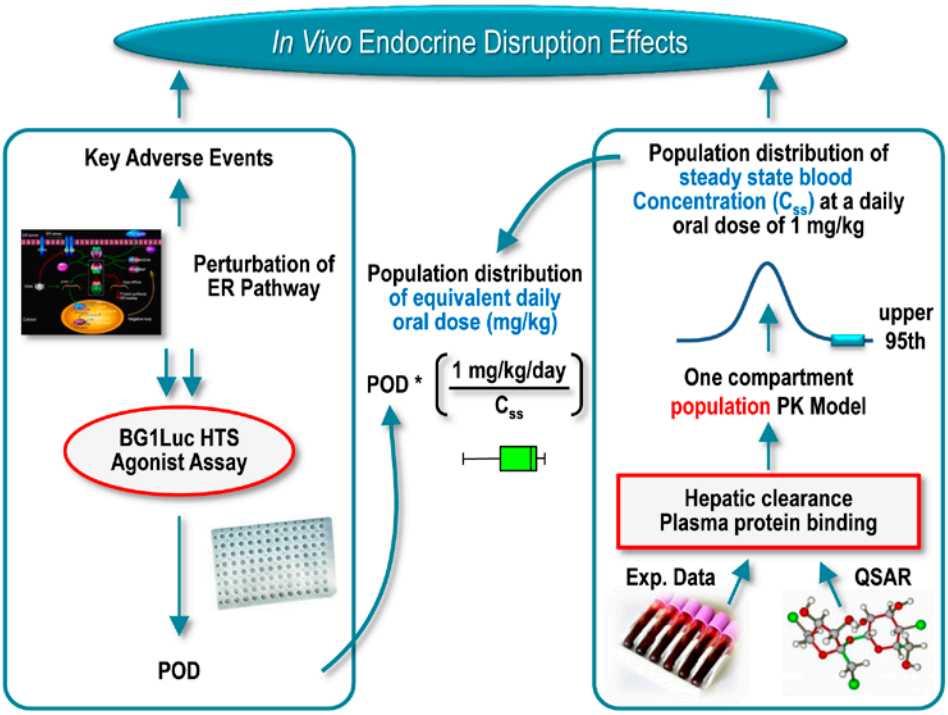
Background and Objective

- The U.S. Environmental Protection Agency (EPA) Endocrine Disruptor Screening Program (EDSP) includes assays that assess chemical effects on estrogen signaling.
- One of these is the *in vitro* BG1Luc estrogen receptor (ER) transactivation assay (BG1Luc). The BG1Luc is accepted internationally for identifying ER agonists and has been adapted to a high-throughput screening (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 and applied a population-based reverse pharmacokinetic modeling approach for each tested chemical.
    - The NICEATM 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 applied to 17β-estradiol (E2) and then was expanded to include 231 more Tox21 compounds, subsets of which are listed in **Tables 3** and **4**.

Figure 1 Use of Pharmacokinetic 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

- Tox21 chemicals tested in the BG1Luc HTS assay were selected for *in vivo–in vitro* extrapolation (IVIVE) analysis. Chemicals were selected based on availability of metabolic clearance and plasma protein binding data (Plowchalk and Teeguarden 2002; Teeguarden et al. 2005; Wetmore et al. 2012, 2013).
    - Out of 58 chemicals selected for use in rat PK models, 11 had uterotrophic assay data (**Table 3**).
    - Out of 232 chemicals selected for use in human PK models, 31 were positive in the BG1Luc HTS assay, of which 23 had human exposure estimates (**Table 4**).

Estimation of Oral Equivalent Doses Using IVIVE

- The POD is the lowest concentration that causes a response that significantly exceeds the background activity level in the BG1Luc HTS assay.
- The daily oral equivalent dose (OED) resulting in a median *in vivo* steady-state blood concentration (Css) equivalent to the POD was estimated using a simple one-compartment population PK model (**Figure 1**). This model assumes that unbound chemical is rapidly cleared so that the bound fraction contributes greatest to the Css and any resulting effects. The OED for a given test substance was then compared to (a) the lowest oral dose that resulted in a significant increase in rat uterine weight or (b) the estimated human exposure.
    - The standard Css for a daily oral dose of 1 mg/kg/day was calculated as:

- To better understand the impact of Fub and CLinvitro on rat OEDs, we did parameter sensitivity analysis by systematically varying Fub for E2 from 0.005 to 1 and CLinvitro for E2 from 0.1 to 100 µl/min per million rat hepatocytes (**Table 2**).

Table 1 Physiological and Biochemical Parameters for E2 Used in the Population PK Model

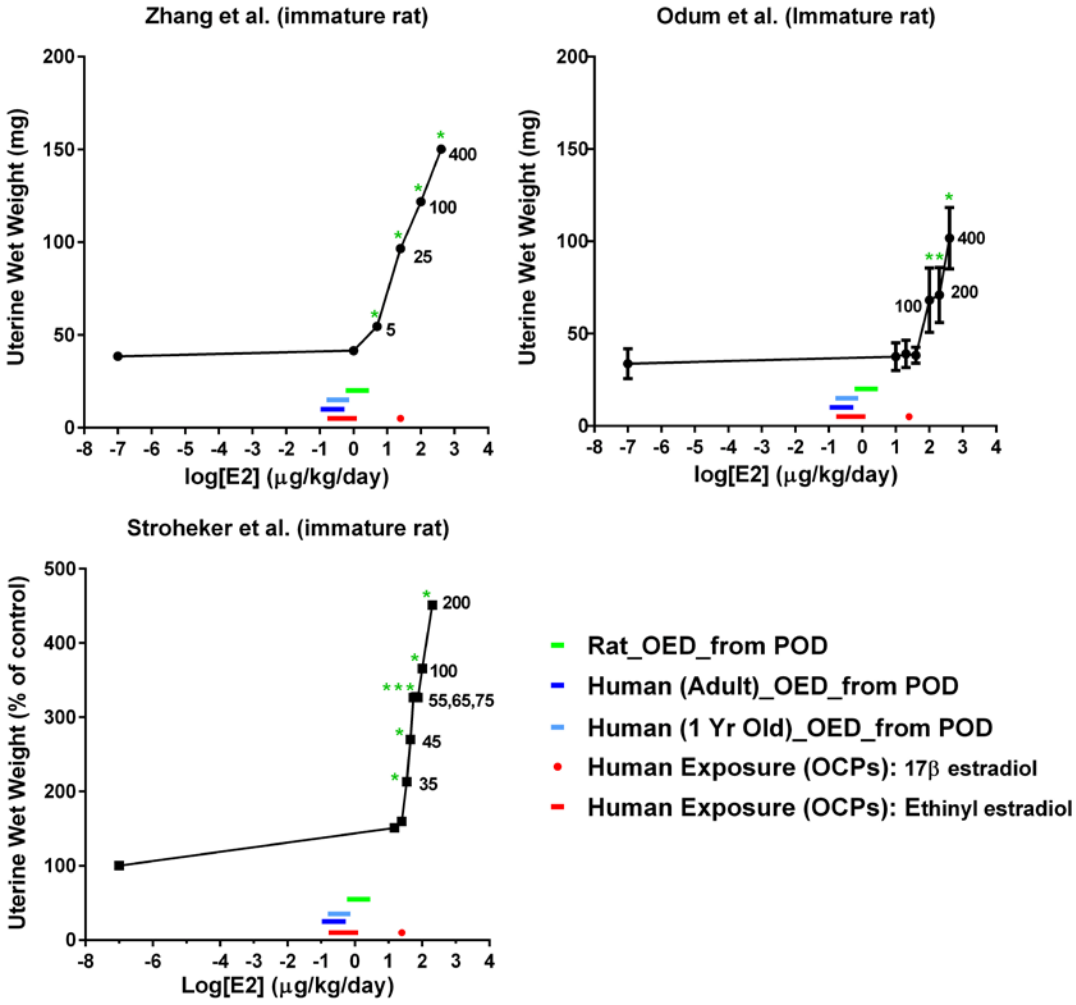
Chemical_Species	GFR (l/h)	Qliver (l/h)	Fub	CLintrinsic (l/h)	CLhepatic (l/h)
E2_Rat	0.08	0.83	0.053[a]	1.00[a]	0.05
E2_Human (Adult)	6.7	90	0.019[a]	150.00[a]	2.76
E2_Human (1 year old)	1.8	21.4	0.021[b]	28.2[b]	0.59

Abbreviations: CLhepatic = hepatic clearance rate; CLintrinsic = intrinsic metabolic clearance rate; E2 = 17β-estradiol; Fub = fraction of unbound plasma protein; GFR = glomerular filtration rate; Qliver = liver blood flow; POD = point of departure.

[a] Plowchalk and Teeguarden (2002)

[b] Adjusted from human adult value.

Figure 2 Uterotrophic Data, Estimated OEDs, and Estimated Human Exposure for E2



Abbreviations: E2 = 17β-estradiol; OED = daily oral equivalent doses; OCPs = oral contraceptive pills; 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). Green 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. The length of the bar covers the 95% confidence interval of the OED estimates.

Table 2 Effect of Varying Fub or CLinvitro on OED Estimates from BG1Luc HTS POD for E2 in Rat[a]

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
Fub = 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
Fub = 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
Fub = 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
Fub = 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
Fub = 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
Fub = 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
Fub = 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
Fub = 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.

Table 3 Selected Tox21 Chemicals with OEDs for Rats Estimated from POD of *In Vitro* Assay and LEDs from Uterotrophic Assays

Chemical	POD (μM) from BG1Luc HTS Agonist Assay	Median OED (mg/kg/day)	Uterotrophic Assay Data: LED (mg/kg/day)[a]	Uterotrophic Assay Data Source	Uterotrophic Assay Data: OECD GL Study?[b]	Ratio: LED/OED
17β-Estradiol	0.000933	0.00133	0.005	Zhang et al. (2012)	Yes	3.8
Bisphenol A	0.137	0.042	200	Matthews et al. (2001)	Yes	4755
Fenarimol	10.233	0.692	200	Andersen et al. (2006)	No	289
Lindane	NEG	NA	NEG (i.p.)	Welch et al. (1971)	Yes	NA
Acetochlor	NEG	NA	NEG	Rollerova et al. (2011)	No	NA
Diethyhexyl phthalate	NEG	NA	NEG	Zacharewski et al. (1998)	No	NA
Fenbuconazole	NEG	NA	NEG (mice)	Ohta et al. (2012)	No	NA
Perfluorooctanoic acid	NEG	NA	NEG/ 0.01 (mice)	Dixon et al. (2012)	No	NA
Permethrin	NEG	NA	NEG/ 800 (s.c.)	Kunimatsu et al. (2002) /Kim et al.	Yes	NA
Simazine	NEG	NA	NEG	Connor et al. (1996)	Yes	NA
Triclosan	NEG	NA	NEG/ NEG (s.c.)	Stoker et al. (2010)/ Louis et al. (2013)	No	NA

Abbreviations: HTS = high-throughput screening; LED = lowest effective dose; NA = not applicable; NEG = negative; OECD GL = guideline-like (GL) protocol modified based on protocol described in Test Guideline 440, "Uterotrophic Bioassay in Rodents", issued by the Organisation for Economic Co-operation and Development (OECD 2007) and EPA guideline documents; OED = daily oral equivalent dose; POD = point of departure.

[a] Except as indicated, studies were conducted with rats with test chemical administered by oral gavage.

[b] Refer to poster **II-3-127 (Ceger et al.)** for a more detailed discussion of what constitutes a guideline-like study.

Table 4 Potential ER Agonists with OEDs for Adult Humans Estimated from POD of *In Vitro* Assay and Estimated Human Exposure

Chemical[a]	**POD (μM) From BG1Luc HTS Agonist Assay**	**OED_median (mg/kg/day)**	**Human Exposure Estimates (mg/kg/day)**[b]	**Ratio: OED/ Highest Exposure Reported or POD/ Serum Level**
17β-Estradiol	0.00093	0.00026	0.00025–0.00083[c]	0.31
Niclosamide	0.010	0.00073	Extremely low[d]	NA
Endosulfan	2.188	0.010	0.000006–0.000102	99.26
HPTE	0.023	0.016	0.00098 μM (serum level)[e]	23.56
Chlorethoxyfos	32.359	0.123	≤ 0.0000006	≥ 205703.28
Bisphenol A	0.137	0.127	0.00048–0.0016[f]	79.32
Thiabendazole	2.362	0.148	0.001026–0.00212	69.81
Fenarimol	10.233	0.291	<0.00002	> 14550
Clofentezine	7.762	0.301	0.000013–0.000052	5783.22
Cyprodinil	3.236	0.463	0.0107–0.0257	18.03
Pyriproxyfen	7.586	0.725	0.007786–0.035166	20.61
Pendimethalin	17.378	1.138	0.00042–0.0014	813.00
Dicofol	70.795	1.631	0.000076–0.000152	10730.12
Norflurazon	13.490	1.730	0.0015–0.00705	245.42
Cyproconazole	30.903	7.476	0.0001	74759.87
Dibutyl Phthalate	18.197	8.853	0.004867133[g]	1818.94
Fluroxypyr-meptyl	30.200	23.715	0.001816–0.01405	1687.93
Carboxin	20.893	24.081	0.0009–0.0029	8303.84
Tebuthiuron	30.200	39.865	0.000012–0.000083	480298.11
Fenhexamid	22.909	43.010	0.014096–0.045219	951.14
Triadimefon	33.884	61.033	0.000607–0.001952	31266.74
Fluazifop-P-butyl	56.234	76.343	0.000814–0.00341	22387.83
Pymetrozine	34.674	135.018	0.0001938–0.000608	222069.70

Abbreviations: ER = estrogen receptor; HPTE = 2,2-Bis(4-hydroxyphenyl)-1,1,1-trichloroethane; HTS = high-throughput screening; NA = not applicable; NEG = negative; OED = daily oral equivalent dose; POD = point of departure.

[a] Chemicals are listed in order of increasing median OED.
[b] From Wetmore et al., 2012, except as noted
[c] Burkman et al., 2011
[d] EPA Reregistration Eligibility Decision (RED) document, EPA 738-R-99-007, 1999
[e] Freire et al., 2013
[f] Vandenberg et al., 2007
[g] CDC, 2013

Results

Rat Data

- The median OED estimate for E2 in rat was 3.8-fold less than the smallest LED in three uterotrophic studies (**Figure 2, Table 3**). The median OED estimates for BPA and fenarimol were 4755 and 289 times less than their corresponding LEDs, respectively. Concordant negative results were found between the BG1Luc assay and uterotrophic studies, indicating good agreement between *in vitro* and *in vivo* ER assays (**Table 3**).
- Sensitivity analyses with E2 as an example showed that the fluctuations in OED estimates are directly related to the CLinvitro and Fub. In combination, Fub and CLinvitro can vary up to 5- or 10-fold from experimental values without significantly impacting the overall OED estimates (within 2-fold) (**Table 2).**

Human Data

- The median OED estimate for E2 in adult humans overlapped with the exposure range of oral contraceptive pills (**Figure 2, Table 4**). For 22 other chemicals, human exposures were well below the OED estimates. Six chemicals (endosulfan, HPTE, bisphenol A, thiabendazole, cyprodinil, and pyriproxyfen) are of highest potential concern due to their low median OED estimates and small differences (<100 fold) between OEDs and human exposure levels (**Table 4**).
- For E2 and BPA, there was <1.5-fold difference in OED estimates between human adults and 1-year-old infants. (Refer to a published SOT poster for BPA data: http://ntp.niehs.nih.gov/iccvam/meetings/sot14/chang-poster.pdf.)

Discussion and Conclusion

- We demonstrated the feasibility of using nominal effective concentrations from an *in vitro* HTS ER transactivation assay in combination with reverse pharmacokinetic modeling to quantitatively predict *in vivo* uterotrophic effects.

- Close agreement between OEDs and *in vivo* LEDs for E2 in rats and humans provides a preliminary indication that our IVIVE approach is valid.
- For the 11 chemicals with uterotrophic data, the OEDs were lower than LEDs in the uterotrophic assay and there was general concordance across the chemicals with negative results. This suggests that combining HTS assay results with IVIVE modeling may provide a conservative hazard prediction.
- The 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, but preliminary analyses suggest that variations in Fub and CLinvitro are well tolerated for OED estimation.
- Incorporation of dosimetry, species- or age- specific pharmacokinetic factors, and exposure estimates is necessary to quantitatively predict *in vivo* effects and for proper interpretation of *in vitro* data for risk assessments.

Acknowledgements

The Intramural Research Program of the National Institute of Environmental Health Sciences (NIEHS) supported this poster. Technical support was provided by ILS under NIEHS contracts N01-ES 35504 and HHSN27320140003C.

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A summary of NICEATM activities at the Ninth World Congress is available on the National Toxicology Program website at http://ntp.niehs.nih.gov/go/41583.