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Predictive Endocrine Testing in the 21st Century Using *in Vitro* Assays of Estrogen Receptor Signaling Responses

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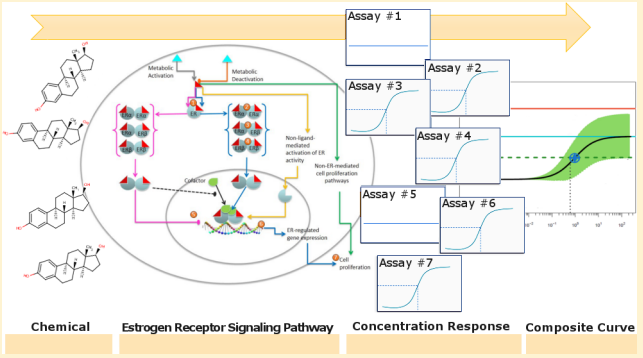
Supporting Information

ABSTRACT: Thousands of environmental chemicals are subject to regulatory review for their potential to be endocrine disruptors (ED). *In vitro* high-throughput screening (HTS) assays have emerged as a potential tool for prioritizing chemicals for ED-related whole-animal tests. In this study, 1814 chemicals including pesticide active and inert ingredients, industrial chemicals, food additives, and pharmaceuticals were evaluated in a panel of 13 *in vitro* HTS assays. The panel of *in vitro* assays interrogated multiple end points related to estrogen receptor (ER) signaling, namely binding, agonist, antagonist, and cell growth responses. The results from the *in vitro* assays were used to create an ER Interaction Score. For 36 reference chemicals, an ER Interaction Score >0 showed 100% sensitivity and 87.5% specificity for classifying potential ER activity. The magnitude of the ER Interaction Score was significantly related to the potency classification of the reference chemicals ($p < 0.0001$). ERα/ERβ selectivity was also evaluated, but relatively few chemicals showed significant selectivity for a specific isoform. When applied to a broader set of chemicals with *in vivo* uterotrophic data, the ER Interaction Scores showed 91% sensitivity and 65% specificity. Overall, this study provides a novel method for combining *in vitro* concentration response data from multiple assays and, when applied to a large set of ER data, accurately predicted estrogenic responses and demonstrated its utility for chemical prioritization.



INTRODUCTION

Estrogen receptors (ER) mediate a vast array of physiological responses through a highly complex network of signaling mechanisms.[1,2] They are essential for sexual development and reproductive function and have important physiological roles in other tissues such as bone and the central nervous system. Estrogen receptors are also involved in pathological processes including breast cancer, endometrial cancer, and osteoporosis.[3−5] Disruption of ER signaling, either directly or through altering estrogen biosynthesis, has been shown to cause adverse effects on reproductive success and fetal development.[3,4,6−9]

Due to the potential consequences of disrupting ER signaling, there is a regulatory need to integrate existing knowledge and to develop prioritization tools capable of testing thousands of chemicals that span a broad range of chemical classes for potential estrogenicity (e.g., industrial chemicals, food additives, cosmetic ingredients, pesticides).[10] Using *in vitro* assays has been proposed as a means to efficiently address the regulatory requirements; however, the complexity of the ER signaling pathway presents multiple challenges. First, there exists two isoforms of ER that are both cell- and tissue-type specific.[11,12] The two forms of ER (ERα and ERβ) are encoded by distinct genes (ESR1 and ESR2) and can have opposing physiological effects. ERα activation generally results in a cell proliferative response, whereas ERβ activation generally results in an antiproliferative and anti-inflammatory response.[13,14] Chemicals that bind to ER will often bind to both isoforms but with differential affinities.[15] Second, a growing body of evidence suggests that many ER ligands are not strictly agonists or antagonists but that weak/partial agonists can act as antagonists under certain conditions.[16] Finally, certain chemical classes are expected to yield false positive or false negative results in some

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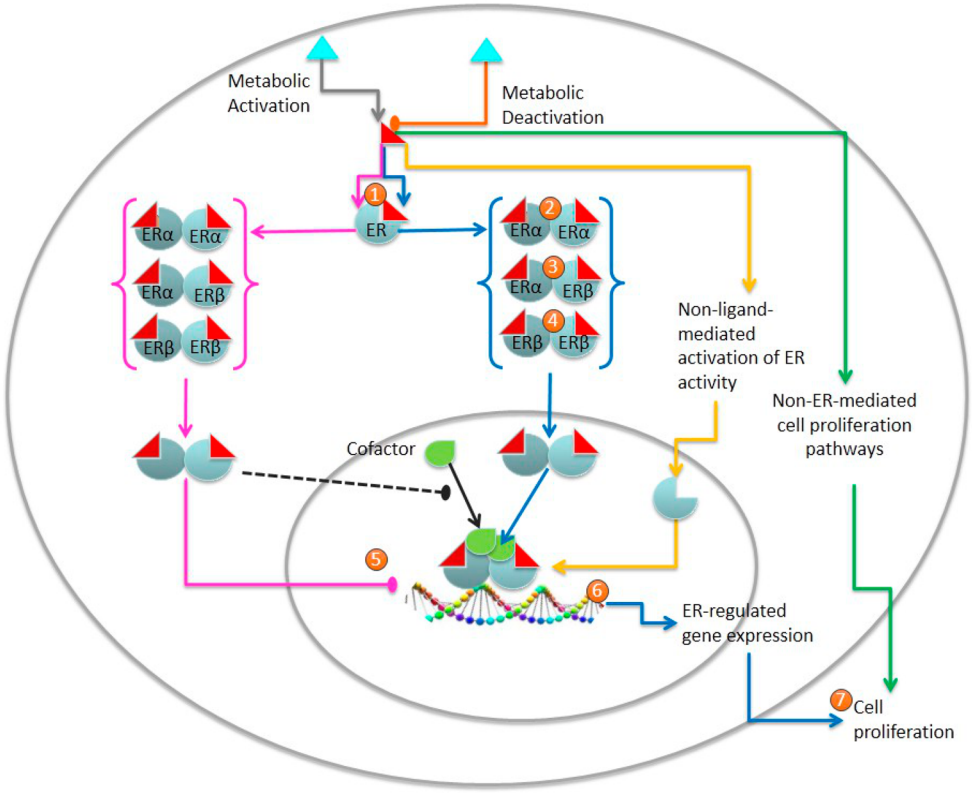

Figure 1. Schematic of ER signaling pathway used to define *in vitro* assay groupings. The available *in vitro* assays measure the ability of a chemical to perturb different molecular events leading to increased or decreased ER activity. The numbers identify the various molecular events measured using the available assays and correspond to the assays in Table 1. Binding group assays, 1; agonist group assays, 2, 3, 4, 6; antagonist group assays, 5; cell growth assay, 7. Cell proliferation was identified as a functional end point of ER activation.

in vitro assays due to assay-specific interference (e.g., issues with solubility, off-target effects, etc.).

To overcome the challenges outlined above, we integrated the results from 13 ER related *in vitro* HTS assays from the EPA ToxCast program into an ER Interaction Score that represents the overall likelihood of a chemical being estrogenic and additionally yields quantitative scores for ERα/ERβ selectivity and agonist/antagonist activity. The ER Interaction Score was constructed based on the canonical ER signaling pathway, for the purpose of building a model that is capable of predicting and prioritizing chemicals for their potential to interfere with the ER signaling pathway. A comparison of the *in vitro*-derived ER Interaction Score with available *in vivo* data, for a subset of reference chemicals, indicates that the approach is capable of accurately predicting estrogenic activity.

MATERIALS AND METHODS

Chemical Selection. The present study was conducted using data from the ToxCast chemical library which consisted of ~1800 unique chemicals. This inventory includes pesticide active and inert ingredients, industrial chemicals, food additives, pharmaceuticals, and other chemical classes. Chemical library information including structures can be found at http://epa.gov/ncct/dsstox/sdf_toxcst.html.

Chemical Quality Control (QC). Chemical samples were commercially procured, diluted in dimethyl sulfoxide (DMSO) to a stock concentration of 20 mM, plated by Evotec (formerly BioFocus DPI, South San Francisco, CA), and shipped to each laboratory running the *in vitro* assays. High-throughput liquid and gas chromatography mass spectrometry determination of sample purity, parent mass, and sample stability in DMSO over time was performed for the phase I chemical inventory (http://www.epa.gov/ncct/toxcast/chemicals.html). The phase II portion of the ToxCast chemical library is currently undergoing similar analytical QC methods in association with the Tox21 project and upon completion will be made publically available. Additional chemical information can be obtained by querying the chemical_id, from File S9 in the SI, in the publicly available DSSTox Structure Browser (http://epa.gov/dsstox_structurebrowser/) which will provide hyperlinks to additional structure-based information in the EPA's ACToR database (http://actor.epa.gov) and the PubChem database (http://pubchem.ncbi.nlm.nih.gov/).

In Vitro Assays. All *in vitro* assays were selected from the ToxCast assay battery. Assays were selected in an effort to maximize coverage of the ER signaling pathway (Figure 1). Further details on all of the following assays are provided in Appendix A of the SI. The NovaScreen (NVS) competitive binding assays for the human (NVS_hER), bovine (NVS_bER), and murine (NVS_mERa) estrogen receptors were developed and run by Caliper Discovery Alliances and Services (Hanover, MD). The NVS_hER, NVS_bER, and NVS_mERa receptor binding assays were conducted on extracts of MCF-7 human breast cancer cells, bovine uterine membranes, and recombinant mouse ERα of the ligand binding domain, respectively. A more complete description of the Novascreen assays and analysis methods is provided in the SI.

The Odyssey Thera (OT) protein-fragment complementation assays (PCA) were developed and run by Odyssey Thera, Inc. (San Ramon, CA). For the assays in the present study, the

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Table 1. ER *in Vitro* Assay Annotation and Model Grouping

map to Figure 1	assay name	assay information	group
1	NVS_NR_hER	human ER binding assay	binding
1	NVS_NR_mERa	murine ERα binding assay	binding
1	NVS_NR_bER	bovine ER binding assay	binding
2	OT_ERaERa_1440_agonist	Odyssey Thera ERα-ERα dimerization in agonist mode after 1440 min	agonist
3	OT_ERa_ERb_1440_agonist	Odyssey Thera ERα-ERβ dimerization in agonist mode after 1440 min	agonist
4	OT_ERbERb_1440_agonist	Odyssey Thera ERβ-ERβ dimerization in agonist mode after 1440 min	agonist
5	Tox21_ERa_LUC_BG1_ Antagonist	ERα luciferase reporter gene assay in human BG-1 ovarian cells in antagonist mode	antagonist
5	Tox21_ERa_BLA_Antagonist_ratio	ERα β-lactamase reporter gene assay in human HEK-293 cells in antagonist mode	antagonist
6	Tox21_ERa_LUC_BG1_Agonist	ERα luciferase reporter gene assay in human BG-1 ovarian cells in agonist mode	agonist
6	Tox21_ERa_BLA_Agonist_ratio	ERα β-lactamase reporter gene assay in human HEK-293 cells in agonist mode	agonist
6	ATG_ERE_CIS	multiplexed ER reporter gene assay using full length receptor in HepG2 cells	agonist
6	ATG_ERa_TRANS	multiplexed GAL4 reporter construct with human ERα ligand-binding domain in HepG2 cells	agonist
7	ACEA_T47D_80h	cell growth using real-time cell analysis in T47D cells at 80 h	cell growth

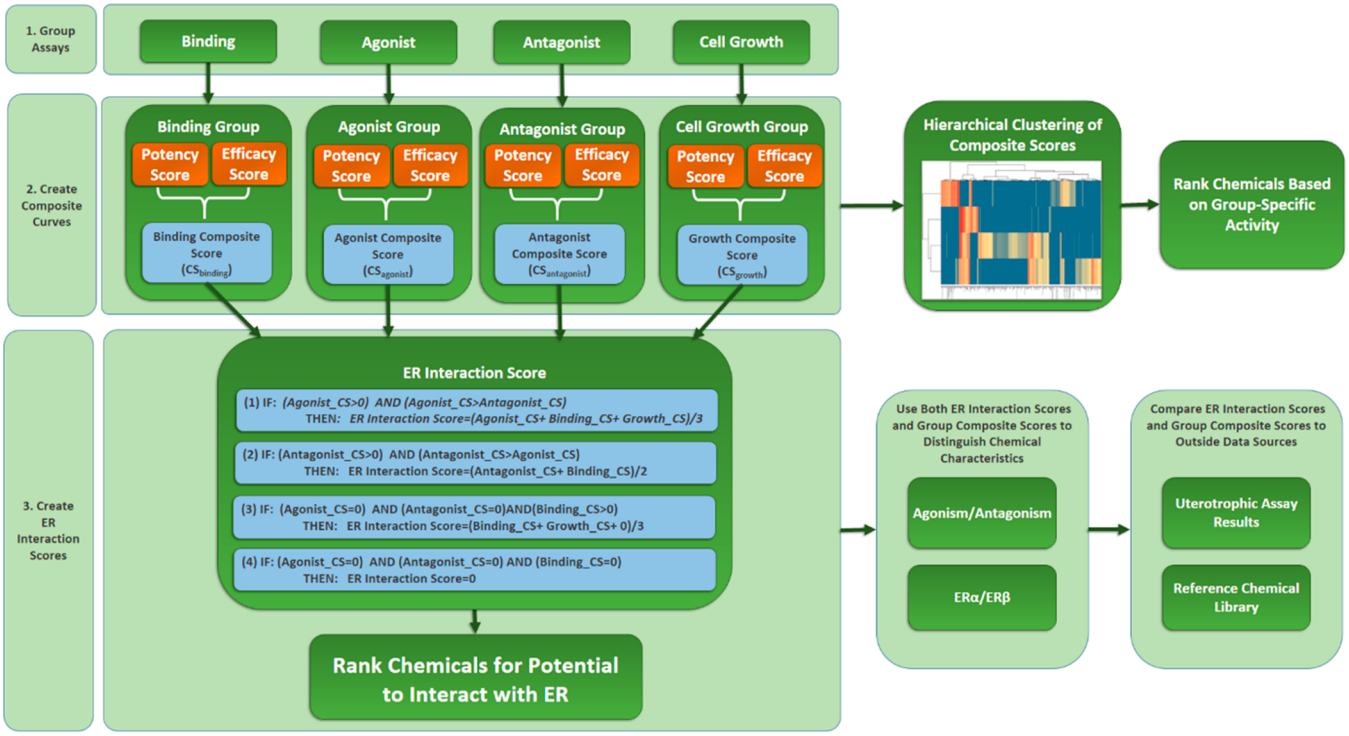

Figure 2. Graphical representation of the ER Interaction Score calculation and downstream analyses performed in the study. (1) The initial step grouped the *in vitro* assays according to the disjunctive logic of the ER signaling pathway (Figure 1). (2) Composite curves were fit to the aggregated data within each group to model the overall concentration response. Composite scores were clustered to identify chemicals with similar activity profiles among groups. (3) ER Interaction Scores were calculated from the composite scores in each group to rank chemicals according to estrogenic likelihood. Individual assays and composite scores were investigated for additional ER characteristics (agonism/antagonist, ERα/ERβ activity).

fused proteins were transiently expressed in human HEK293T kidney cell lines and target ERα and ERβ homo- and heterodimerization. Assays were run across three dimerization conditions (ERα-ERα, ERα-ERβ, ERβ- ERβ) and were designated OT_ERaERa_1440, OT_ERaERb_1440, and OT_ERbERb_1440, respectively. A more complete description of the Odyssey Thera assays and analysis methods is provided in the SI.

The Attagene ER assays (ATG_ERa_TRANS, ATG_ERE_-CIS) consisted of both a cis- and trans-system and were developed and run by Attagene, Inc. (Research Triangle Park, NC). Both of these assays are ER reporter gene assays. A major difference between the cis- and trans-system is that in cis activities of endogenous transcription factors are measured, whereas the trans assay evaluates changes in activities of the exogenous, chimeric hERα-Gal4 that regulates transcription of a reporter sequence. Additional details on the Attagene assays and analysis methods are provided in the SI.

The ACEA assay (ACEA_T47D_80 h_Positive) was performed by ACEA Biosciences, Inc. (San Diego, CA). The assay utilizes the xCELLigence Multi-E-Plate system that measures time-dependent cell growth using impedance using the ER-responsive T-47D breast cancer cells. To make cross platform comparisons, we selected a late time point (80 h) that appeared to capture the full growth potential of active chemicals and was tested across all chemical libraries in the

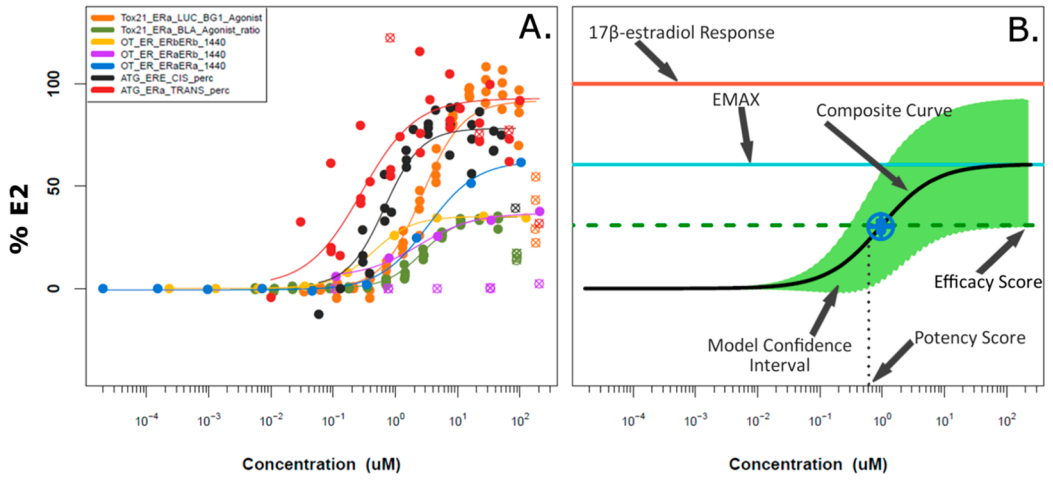

Figure 3. Example of the *in vitro* assay aggregation and fitting of the composite concentration response curve. The present example shows how the $CS_{agonist}$ model was fit for Bisphenol A. (A) Linearly transformed, concentration response curves for all available *in vitro* assays included in the agonist group for Bisphenol A. (B) Composite concentration curve of all the individual concentration response curves from the agonist group for Bisphenol A. The lower window of the model confidence interval is used to calculate the efficacy score. The first concentration for which the lower window of the confidence interval does not overlap with zero is assigned as the LEC. The percentage across all tested concentrations that the LEC occurs is the Potency Score. If both the Potency and the Efficacy Score > 0, the mean of the Potency and Efficacy score is assigned as the Composite Score for that group.

cell growth assay. Additional details on the ACEA assay and analysis methods are provided in the SI.

The Tox21 ER assays were run by the National Center for Advancing Translational Sciences (NCATS) at the National Institutes of Health (NIH) in Bethesda, Maryland and consist of two high-throughput reporter gene assays run in both agonist (Tox21_ERa_BLA_agonist, Tox21_ERa_LUC_BG1_agonist) and antagonist mode (Tox21_ERa_BLA_antagonist, Tox21_ERa_LUC_BG1_antagonist). Additional details on the Tox21 assays and analysis methods are provided in the SI.

Data Processing. All assay responses were normalized to 17β-estradiol, except those in the antagonist group, which were normalized to 4-hydroxytamoxifen. The normalized data from all chemicals for each assay were fit using nonlinear least-squares regression to a Hill-model in a separate analysis. A diminished response, observed at higher concentrations for some chemicals, is characteristic of cytotoxicity. This has been confirmed using assays that measure ATP or expert inspection performed either during assay development or run in parallel with the assays shown. Details on the model fitting and filtering for cytotoxicity and outliers, where applicable, are outlined in the SI.

After fitting the experimental data for each chemical and assay combination, the model was used to generate a series of 1000 interpolated data points at standardized concentrations. This serves to create a consistent set of concentrations across all assays within a group and also ensures that each assay is weighted equally regardless of the number of concentrations or replicates tested for a given assay. Next, the assay-specific interpolated data points were organized according to their assigned group within the ER pathway (Figure 1 and Table 1). A schematic representing the data processing and analysis workflow is provided in Figure 2. The NVS_hER, NVS_mERa, and NVS_bER binding assays were grouped together because both agonist and antagonist activity are detectable and indistinguishable. Agonist assays, OT_ERaERa_1440, OT_ERaERb_1440, OT_ERbERb_1440, Tox21_ERa_BLA_agonist, Tox21_ERa_LUC_BG1_agonist, ATG_ERa_TRANS_perc, and ATG_ERE_CIS_perc, were grouped together. Antagonist assays, Tox21_ERa_BLA_antagonist and Tox21_ERa_LUC_BG1_antagonist, were grouped together (Figure 2). Because the ACEA_T47D_80h assay responds to growth inducing chemicals other than estrogens,[17] it was given a group of its own. In order to minimize the fitting of variability/noise specific to the platform on which the assay was conducted, the interpolated data from the different assays within groups were linearly transformed. This removed some of the assay-specific noise, so that the remaining variability in responses was more likely to be from biological variability. Information on how the linear transformation was conducted can be found in the SI, and the effect of the linear transformation across all chemicals is illustrated in Figure S1 in the SI.

Composite Concentration Response Curves. The interpolated, aggregated, and linearly transformed data for each of the assigned groups was fit to a single, composite concentration curve using a Hill model and nonlinear least-squares regression in the open-source statistical software, R and the *sfsmisc* package.[18,19] A representation of a composite model can be seen in Figure 3. The four-parameter Hill equation is represented as

$$Y = T - \frac{(T - B)}{1 + \left(\frac{X}{AC50}\right)^{W}}$$

The top (T) was constrained to ±20% of the maximum response observed across all assays in the group in order to minimize convergence errors. The bottom (B) was constrained to 0, and the hillslope (W) was constrained to 1. X is the chemical concentration, and $AC50$ is the concentration at which 50% activity occurs. Lastly, Y is the resulting response value. The constraint for B was selected because the model was allowed to fit outside of the concentration range. This means that as X approaches 0, B should also approach 0. The constraint of 1 was selected for W to encourage convergence when responses varied within a group. Bootstrapping was performed in order to determine the uncertainty around the

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composite model, this was done by generating 100 composite models by sampling individual assays in a group with replacement. The size of each sample was consistent with the number of unique assays in the group. For example, there were 7 assays in the agonist group, so 7 assays were sampled with replacement 100 times and fit to a total of 100 composite curves. A 99% confidence interval of the bootstrapped samples was calculated at each of the 1000 interpolated concentrations along the composite models. This is advantageous because responses with more agreement at a given concentration will have narrower confidence intervals compared to discordant responses at a given concentration (Figure 3). Because the growth group contained only a single assay, a 90% prediction interval was calculated from the sum of squared errors. In order to obtain uncertainty estimates when there were not enough data points available to calculate the prediction interval within the growth group, interpolated data points were added to the response but were weighted so that they did not impact the error calculation.

Calculation of the ER Interaction Score. An Efficacy Score (%) was calculated from the maximum value of the lower-window of the bootstrapped confidence interval. If the window does not overlap with zero, then a chemical was considered to have a statistically significant response and given an Efficacy Score > 0. If the maximum value of the lower-window overlaps with 0, then the Efficacy Score will equal 0.

The first concentration at which the lower window of the confidence interval was greater than zero was annotated as the lowest effective concentration (LEC) (μM). The Potency Score (%) was obtained by taking the lowest and highest concentrations across all chemicals as 100% and 0%, respectively, and calculating the percentage at which the LEC occurs. If the lower-window of the confidence interval never increased above 0, then the Potency Score was set to 0. Separate ERα and ERβ Potency Scores were calculated using the AC50s from the OT_ERaERa_1440 and the OT_ERbERb_1440, respectively.

If both the Potency and Efficacy Scores were >0, the means of the Potency Scores and the Efficacy Scores were assigned as the Composite Score (CS) for the chemical-group. Otherwise the CS is set to 0. The final CS for each chemical-group was calculated by scaling the value to the maximum CS within each group, so that when comparing across groups, each group is weighted equally. A graphical representation of these parameters is shown in Figure 3. If all individual assays within a group were active for a chemical, but the composite score was 0 due to the uncertainty around the individual assay responses, then the chemical was flagged as a chemical of interest in the output file.

The ER Interaction Score is calculated based on the following logic

$$\text{IF: } (CS_{agonist} > 0) \text{ AND } (CS_{agonist} > CS_{antagonist})$$
$$\text{THEN: ER Interaction Score} = (CS_{agonist} + CS_{binding} + CS_{growth})/3 \quad (1)$$

$$\text{IF: } (CS_{antagonist} > 0) \text{ AND } (CS_{antagonist} > CS_{agonist})$$
$$\text{THEN: ER Interaction Score} = (CS_{antagonist} + CS_{binding})/2 \quad (2)$$

$$\text{IF: } (CS_{agonist} = 0) \text{ AND } (CS_{antagonist} = 0) \text{ AND } (CS_{binding} > 0)$$
$$\text{THEN: ER Interaction Score} = (CS_{binding} + CS_{growth} + 0)/3 \quad (3)$$

$$\text{IF: } (CS_{agonist} = 0) \text{ AND } (CS_{antagonist} = 0) \text{ AND } (CS_{binding} = 0)$$
$$\text{THEN: ER Interaction Score} = 0 \quad (4)$$

where $CS_{agonist}$ is the agonist Composite Score, $CS_{antagonist}$ is the antagonist Composite Score, $CS_{binding}$ is the binding Composite Score, and CS_{growth} is the growth Composite Score. The 0 term is added to eq 3 because the binding assays cannot distinguish agonist from antagonist activity; therefore, we use the 0 term to represent the value from either the $CS_{agonist}$ or $CS_{antagonist}$.

Hierarchical Clustering. Two-way hierarchical clustering was performed using the "pheatmap" package in statistical software, R.[19,20] Two separate heatmaps were generated, one with all chemicals, and one with only chemicals with Composite Scores >0 for at least one group (binding, agonist, antagonist, or growth). Clustering was performed using Euclidean distance and Ward's method.

Model Evaluation. The impact of the linear transformation was determined by comparing distributions of R^2 values across chemicals in the agonist and antagonist groups, respectively, with and without the linear transformation. Statistical significance was determined using a one-tailed Wilcoxon test, and a false-discovery rate correction was applied to correct for multiple comparisons ($q < 0.05$).[21] In addition, the analysis was repeated with only chemicals that produced ER Interaction Scores > 0.

A comparison of the ER Interaction Score was made to 36 ER reference (both positive and negative) chemicals. A list of reference chemicals and the details regarding the selection and curation of the reference chemical list can be found in the SI, and a list of the reference chemicals is available in Table S1 in the SI. ER Interaction Scores were binned by reference chemical categories (strong, strong-moderate, moderate, weak, very weak/agonist (metabolism required), or negative), and the distributions were displayed as box plots. Each group of binned ER Interaction Scores was tested for significance using the Mann−Whitney U test. A binned group was considered statistically significantly different from another group if the Mann−Whitney U test resulted in a $p < 0.1$. The overall trend was tested for significance using a Kruskal−Wallis test.

The association between the ER Interaction Scores and *in vivo* rodent estrogenicity was determined using a subset of chemicals with available *in vivo* uterotrophic data from the Organisation of Economic Co-operation and Development (OECD) guideline and Endocrine Disruptor Screening Program (EDSP) T1S validation studies. Chemical activity from these studies was classified as either being active or inactive for agonist or antagonist activity based on changes in rodent uterine weight and was consistent with Rotroff et al.[22](see the SI).

Comparisons of the ER Interaction Score to both the ER reference chemical set and the uterotrophic data was evaluated based on sensitivity (the ability to correctly classify positives) and specificity (ability to correctly classify negatives). Sensitivity is measured as the proportion of chemicals with ER Interaction

Table 2. Top 25 Chemicals with Highest ER Interaction Scores

CAS RN	chemical name	composite score				ER interaction score	ERα dimerization potency score	ERβ dimerization potency score
		binding	agonist	antagonist	growth			
82640-04-8	raloxifene hydrochloride	86.18	0.00	99.89	0.00	93.04	70.23	20.67
57-91-0	17α-estradiol	94.96	82.81	0.00	76.58	84.78	70.82	75.22
50-28-2	17β -estradiol[a]	83.21	100.00	0.00	63.65	82.28	68.42	67.60
68392-35-8	4-hydroxytamoxifen	82.77	0.00	81.00	0.00	81.89	68.69	23.25
57-63-6	17α-ethinylestradiol	98.63	85.10	0.00	50.68	78.14	69.85	65.41
84-16-2	meso-hexestrol	100.00	77.33	0.00	57.08	78.14	67.48	68.50
56-53-1	diethylstilbestrol	97.34	77.58	0.00	48.14	74.35	70.88	77.83
50-28-2	17β-estradiol[b]	99.12	82.99	0.00	35.82	72.65	71.15	76.66
50-41-9	clomiphene citrate	80.30	0.00	59.86	0.00	70.08	89.81	65.64
53-16-7	estrone	89.80	59.02	0.00	56.42	68.41	46.94	49.88
486-66-8	daidzein	55.55	49.13	0.00	100.00	68.23	55.87	74.75
50-27-1	estriol	82.79	61.64	0.00	51.46	65.30	55.29	38.66
1478-61-1	bisphenol AF	87.10	48.82	0.00	57.58	64.50	47.15	57.06
105624-86-0	5HPP-33	74.29	54.74	33.42	62.54	63.85	100.00	86.82
131-55-5	2,2′,4,4′-tetrahydroxybenzophenone	70.22	68.57	0.00	51.58	63.46	54.82	60.68
2971-36-0	2,2-bis(4-hydroxyphenyl)-1,1,1-trichloroethane (HPTE)	37.12	0.00	82.06	57.07	58.75	85.53	59.80
77-40-7	bisphenol B	37.47	0.00	79.78	48.13	55.13	41.53	55.96
80-05-7	bisphenol A	39.85	0.00	66.99	49.88	52.24	32.15	43.76
84852-15-3	4-nonylphenol branched	62.69	0.00	38.21	52.07	50.99	56.91	60.40
129453-61-8	fulvestrant	0.00	100.00	0.00	17.97	50.00	5.77	5.67
94-18-8	benzylparaben	36.27	0.00	70.54	40.07	48.96	35.37	44.06
10161-33-8	17beta-trenbolone	46.17	0.00	54.59	44.21	48.32	0.00	28.14
68-22-4	norethindrone	47.11	0.00	56.58	40.52	48.07	20.24	18.43
27955-94-8	4,4′,4″-ethane-1,1,1-triyltriphenol	19.84	0.00	59.25	52.75	43.95	54.89	56.42
76-87-9	triphenyltin hydroxide	0.00	87.54	0.00	0.00	43.77	NA	NA

[a]Tested at concentrations up to 3.8 μM. [b]Tested at concentrations up to 200 μM.

Scores > 0 which were also classified as active in either the ER reference chemicals or the uterotrophic analysis. Specificity is measured as the proportion of chemicals with ER Interaction Scores equal to 0 which were also classified as inactive in either the ER reference chemicals or the uterotrophic analysis. Balanced accuracy refers to the mean of the sensitivity and specificity.

RESULTS

Breakdown of Group-wise ER Assay Activity. Experimental data used in the analysis was obtained from a total of 1814 chemicals tested in the 13 assays (Table 1). Assays were split into four groups depending on the type of ER-dependent activity reported: receptor binding, agonist mode transactivation, antagonist mode transactivation, and cell growth. A CS value was calculated for each chemical in each of the four groups. Overall, 47 (2.6%), 212 (11.7%), 107 (5.9%), and 253 (13.9%) chemicals produced CS values > 0 for $CS_{binding}$, $CS_{agonist}$, $CS_{antagonist}$, and CS_{growth}, respectively. Chemicals that produced CS > 0 in only a single group totaled 13, 117, 67, and 142 for $CS_{binding}$, $CS_{agonist}$, $CS_{antagonist}$, and CS_{growth}, respectively. The majority (74.4%) of chemicals do not show activity in any group. Hierarchical clustering of chemicals with a CS > 0 is provided in Figure S2 in the SI.

The group-wise CS values were combined into an overall ER Interaction Score where a total of 323 (17.8%) chemicals possessed an ER Interaction Scores > 0. The 15 chemicals with the highest ER Interaction Scores are shown in Table 2. These include both known agonist and antagonist chemicals. 17α-Estradiol, raloxifene hydrochloride, 4-hydroxytamoxifen, and 17β-estradiol had ER Interaction Scores > 80. Most of the chemicals in this subset had both high ERα and ERβ Potency Scores indicating strong activity for both isoforms; however, some chemicals displayed slight selectivity.

Agonist and Antagonist Comparisons. A broad spectrum of ER activity spanning from potent agonist to potent antagonist was observed among the chemicals tested (Figure 4). Estrogens 17α-estradiol, 17β-estradiol, 17α-ethinylestradiol, 17α-estradiol, and diethylstilbestrol were among the chemicals with the highest $CS_{agonist}$ values. Phytoestrogens apigenin, daidzien, and genistein also showed relatively high $CS_{agonist}$ values of 59.91, 49.13, and 44.33, respectively. The agonist activity can be further subdivided for ERα and ERβ selectivity based on the ERα and ERβ Potency Scores.

For antagonist activity, the four highest $CS_{antagonist}$ values were associated with pharmaceutical compounds (raloxifene hydrochloride, 4-hydroxytamoxifen, clomiphene citrate, and fulvestrant) (Figure 4). The highest $CS_{antagonist}$ value among nonpharmaceuticals was for triphenyltin hydroxide at 87.54. Although efforts were made to minimize the impact of cytotoxicity on the $CS_{antagonist}$ values, it is likely that many of the chemicals with a weak $CS_{antagonist}$ value are causing a loss of signal through cytotoxicity instead of antagonizing ER activation. Methods to better characterize loss-of-signal *in vitro* assays and reduce confounding from cytotoxicity is an ongoing area of research.

Eleven chemicals produced both $CS_{agonist}$ and $CS_{antagonist}$ > 0 (5HPP-33, 4-dodecylphenol, silwet L77, 25-di-*tert*-butylben-

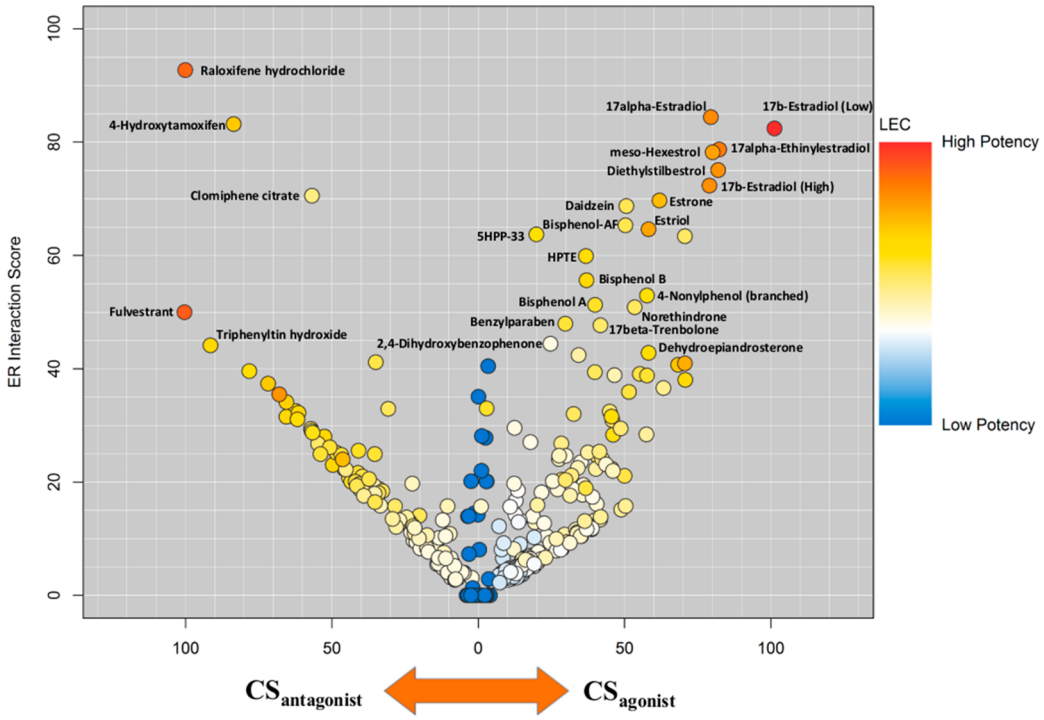

Figure 4. Volcano plot of agonist and antagonist activity for all chemicals. Agonist and antagonist CS values were plotted as a function of the ER Interaction Score on the *y*-axis and or $CS_{agonist}$ and $CS_{antagonist}$ on the *x*-axis. Chemicals with high ER Interaction Scores and high $CS_{agonist}$ fall to the upper right quadrant, whereas chemicals with high ER Interaction Scores and high $CS_{antagonist}$ are located in the upper left quadrant. The colors are determined by the potency of the LEC value. Some chemicals may have a high ER Interaction Score driven partially by their efficacy relative to another chemical's more potent response. The chemicals in the middle of the *x*-axis with ER Interaction Score > 0 had no significant activity in either the agonist or antagonist groups but were active in the binding assay group.

zene-14-diol, surinabant, hydramethylnon, 5-chlorosalicylanilide, metconazole, nilutamide, chlorophacinone, bicalutamide). For this subset, 5HPP-33 was the only chemical that also had significant $CS_{binding}$, and five had significant CS_{growth} (5HPP-33, 4-dodecylphenol, silwet L77, surinabant, hydramethylnon). The CS values for this subset of chemicals ranged from 10.78−54.74 and 12.71−55.38 for $CS_{agonist}$ and $CS_{antagonist}$, respectively.

ERα and ERβ Selectivity. The agonist activity of the receptor was further investigated for ERα ERβ selectivity based on the ERα and ERβ Potency Scores. A total of 12 chemicals demonstrated statistically significantly increased ERα Potency Scores relative to ERβ. However, only 3 of these chemicals had overall $CS_{agonist} > 0$ (2,2′,6,6′-tetrachlorobisphenol A, 4-*tert*-butylphenyl salicylate, 2,2-bis(4-hydroxyphenyl)-1,1,1-trichloroethane). In contrast, a total of 64 chemicals demonstrated statistically significantly increased ERβ Potency Scores relative to ERα. Of these chemicals, 32 had overall $CS_{agonist} > 0$. Most of the chemicals that displayed increased ERβ Potency Scores produced relatively low ER Interaction Scores, with only 4 chemicals producing ER Interaction Scores >20 (dehydroepiandrosterone, quercetin, fenhexamid, silwet L77). Selective estrogen receptor modulator (SERM) chemicals, raloxifene and tamoxifen, have been identified as being selective for ERα.[23] Both of these had significantly elevated ERα Potency Scores relative to ERβ in the present model ($p < 0.01$), although they produced a $CS_{agonist}$ score of 0. The results of the ERα and ERβ comparison for $CS_{agonist}$ are further described in the SI and illustrated in Figure S3 in the SI.

Comparison to ER Reference Chemicals. The chemical library screened through the *in vitro* assays contained 36 reference compounds that were used to assess the performance of the model (Figure 5a and 5b). Reference chemicals were classified as strong (3 chemicals), strong-moderate (1 chemical), moderate (4 chemical), weak (14 chemical), very weak (3 chemicals), and negative (8 chemicals).[24] One chemical, methoxychlor, was classified as very weak and requires metabolic activation. An additional three chemicals (raloxifene hydrochloride, clomiphene citrate, and fulvestrant) were included as antagonists. Of the 28 chemicals classified as positive from strong to very weak, all 28 possessed ER Interaction Scores > 0. Of the eight chemicals classified as negative, seven had ER Interaction Scores of zero. Progesterone was the only negatively classified chemical that produced an ER Interaction Score > 0. The ER Interaction Score for progesterone was 8.09, which was one of the lowest of all the chemicals with ER Interaction Scores > 0 (Figure 5a). Using an ER Interaction Score > 0 as the cutoff, the overall classification accuracy was 97% (35/36) with a sensitivity and specificity of 100% and 87.5%, respectively.

To evaluate the relationship of the ER Interaction Scores relative to the potency class, the distributions of ER Interaction Scores were compared for each reference group (Figure 5b). Overall, a Kruskal−Wallis test yielded a strong association between the reference chemical classifications and the ER Interaction Scores ($p < 0.0001$). Among pairwise contrasts, the negative group was statistically significantly different from all other groups ($p < 0.1$), and there was a significant difference between the weak group and the moderate and strong groups ($p < 0.1$).

Comparison to in Vivo Uterotrophic Assay. Uterotrophic assays are designed to measure estrogenic activity through increased uterine weight in immature or ovariectom-

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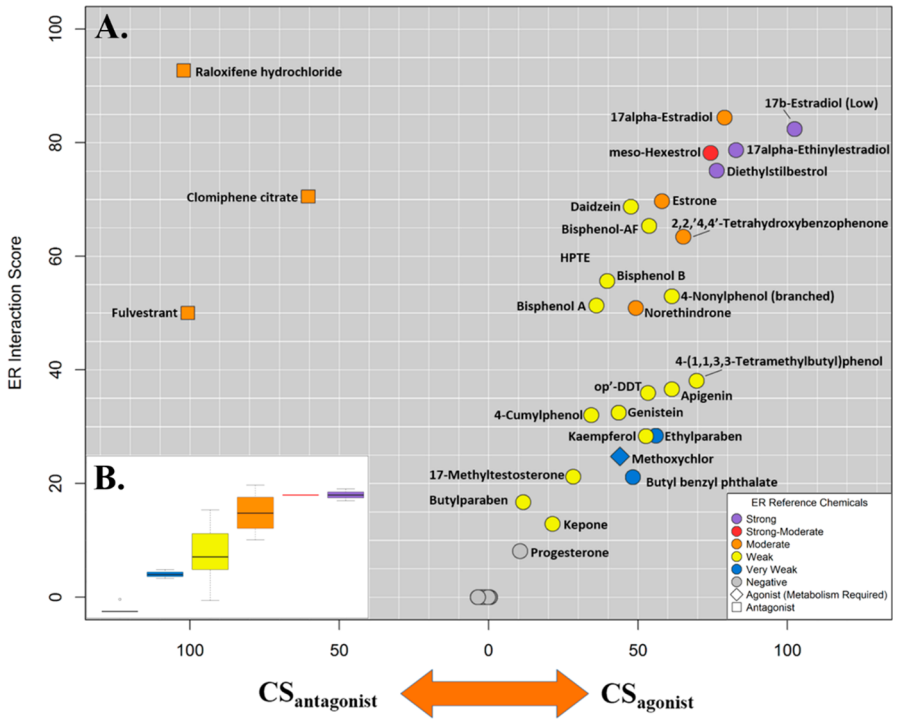

Figure 5. Concordance of ER Interaction Scores and reference chemical classification. (A) Volcano plot of ER Interaction Scores as a function of $CS_{agonist}$ and the $CS_{antagonist}$ values for a subset of reference chemicals with defined levels of receptor activity. Reference chemical classifications were determined by expert review of the literature and NICEATM consensus. (B) Distributions of ER Interaction Scores were evaluated within each of the reference classes. The *y*-axis is the ER Interaction Score, and the *x*-axis are different reference chemical classifications. Reference chemicals were classified as strong, strong-moderate, weak, very weak, or negative according to expert literature review and in consensus with NICEATM evaluations. An overall association between ER Interaction Scores and reference chemical classifications was statistically significant ($p < 0.0001$). A more detailed version of this figure with statistical tests for each group is presented in Figure S8 in the SI.

ized rats.[25] Data from *in vivo* uterotrophic studies were available for a total of 45 chemicals with 23 negatives and 22 actives (12 agonists, 2 antagonists, 8 with both agonist and antagonist activity). Within this 45 chemical subset, 17 chemicals produced ER Interaction Scores of 0, with 2 false negatives (atrazine and disulfiram). In this sense, false negative refers to a chemical with an ER Interaction Score of 0 and classified as having uterotrophic activity. The two false negatives, atrazine and disulfiram, were both classified as antagonists in the uterotrophic assay. A total of 8 of the 28 compounds with ER Interaction Scores > 0 were false positives based the uterotrophic results. In this sense a false positive refers to a chemical with an ER Interaction Score > 0 and is classified as having no uterotrophic activity in the present analysis. However, besides ethylparaben, the remaining 7 of these false positive chemicals (4-nonylphenol, dipentyl phthalate, flutamide, 2-naphthalenol, captafol, dibutyl phthalate, dihexyl phthalate) fall within the lowest 9 ER Interaction Scores > 0 (Figure S4 in the SI). Twenty out of the 28 chemicals with ER Interaction Scores > 0 were classified as true positives resulting in an overall sensitivity and specificity of 91% and 65%, respectively.

DISCUSSION

The present study describes a novel approach for incorporating data from multiple *in vitro* assays into an integrated quantitative score that reflects a chemical's perturbation of a specific signaling pathway. An integrated approach was necessary because each of the individual *in vitro* screening assays have specific limitations or measure only pieces of the larger pathway. Of particular concern is a potentially high false negative rate associated with relying on a single *in vitro* assay since classifying an active chemical as nonactive could result in contained use of chemicals that could cause adverse outcomes (i.e., be true endocrine disruptors). This would outweigh the added cost of subsequent testing for the false positive chemicals (in vitro positive, in vivo negative). The ER pathway is an ideal candidate for developing an integrated assay approach because data are available from multiple *in vitro* assays that assess different aspects of the pathway across a large number of chemicals. Furthermore, a relatively well-established list of reference chemicals and *in vivo* data are available to validate model predictions. We previously explored whether endocrine-related *in vitro* assays were capable of classifying chemicals similarly to EDSP Tier 1 assays for androgen, estrogen, thyroid, and steroidogenesis modes of action (MOA).[22] The purpose of the classification analysis in Rotroff et al. 2013 was to compare and identify MOA for which a predictive model would be mostly likely to succeed. The estrogenic MOA was identified as having a high classification rate, and the model described here aims to build upon the findings in Rotroff et al. 2013 to create a predictive and quantitative model for the purposes of chemical prioritization. In addition, the classification model developed in Rotroff et al. 2013 suggests that estrogenic chemicals are classified more accurately when using more than a single *in vitro* assay, suggesting a more "weight-of-evidence" or consensus approach using multiple *in vitro* assays would be most likely to succeed.[22]

The *in vitro* assay grouping used to derive the ER Interaction Score was based on their functional end point relative to the

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signaling pathway. It is difficult to determine which individual assays are essential for developing an accurate ER Interaction Score. However, it is clear that each assay has its own set of uniquely active chemicals, and the overall ER Interaction Score is not significantly driven by any single assay (see the SI). Separating assays into functional groups (binding, agonist, antagonist, and growth) provides the advantage of enhancing the strengths of the individual assays while down-weighting the impact of their weaknesses. This is because groups can be incorporated into the model in a way that maximizes predictive capabilities. For example, the ER-dependent cell proliferation assay detects growth-promoting activity from multiple pathways including responses to nonestrogenic steroid hormones.[17] Daidzein, a phytoestrogen, was detected as having the highest CS_{growth} (Table 2). It is unlikely that this chemical is more active than chemicals such as 17β-estradiol and 17α-ethinylestradiol and is possibly due to crosstalk with other cell-growth related signaling pathways in this assay. However, when interpreted in the context of the other CS groups, its overall ER Interaction Score is less than 17β-estradiol and 17α-ethinylestradiol, which is likely more representative of daidzein's known ER activity (Table 2). Similarly, ER binding assays are susceptible to false positives from detergent compounds by displacement of the ligand by receptor denaturation. Many of these false positive signals can be identified by their presence in assay-specific clusters, while the cluster that includes active compounds for all three of these groups includes known estrogenic compounds such as estrone, daidzein, and 17α-ethinylestradiol (Figure S2 in the SI).[26−29] Nonetheless, the assays in the binding group were only tested up to 50 μM. Therefore, some weakly active chemicals may be undetected with these assays alone, so chemicals that are weakly active in the agonist and proliferation assays, tested to higher concentrations, are also likely to be true positives.

A comparison of the ER Interaction Scores from well-established ER reference chemicals[24] suggested that the approach identifies potential ER active chemicals with a high sensitivity (100%) and moderate specificity (87.5%) for an overall balanced accuracy of 93.8%. Furthermore, the model's ER Interaction Scores successfully ranked chemicals according to their reference classifications demonstrating a proportional association between the ER Interaction Score and the degree of ER activity. When applied to a broader set of chemicals with *in vivo* uterotrophic data, the ER Interaction Scores showed 91% sensitivity and 65% specificity, for an overall balanced accuracy of 78%. The studies used in this analysis were all performed to OECD guidelines allowing for relatively consistent comparisons across chemicals.[22] Two chemicals, atrazine and disulfiram, were classified as false negatives, having antagonistic uterotrophic activity and ER Interaction Scores of 0. Disulfiram was detected as causing decreased uterine weight at 100 mg/kg/day but was negative in both *in vitro* antagonist assays. Although the sensitivity of the model was 91%, the 13 assays used to construct the ER Interaction Score do not represent all mechanisms for which a chemical can causes changes in uterine weight. For example, atrazine is known to suppress estrogen synthesis through the inhibition of aromatase and does not act through the estrogen receptor; therefore, it would not have been expected to be detected in these assays.[30] In addition, proliferative impacts on uterine weight are largely ERα mediated,[31] so chemicals with high ER Interaction Scores due to predominantly ERβ activation may produce a false positive for the uterotrophic comparison. However, no chemicals meeting this criterion were available for comparison in the uterotrophic analysis. Furthermore, the vast majority of chemicals with significantly elevated ERβ Potency Scores relative to ERα Potency Scores had overall low ER Interaction Scores.

The ER Interaction Score provides a metric for ranking chemicals based on their potential to interact with the ER pathway. The individual composite scores and *in vitro* assay characteristics can then be examined to investigate the specific activity associated with each chemical (e.g., agonist/antagonist, ERα/ERβ). A major goal of this approach is to enable screening of thousands of untested environmental chemicals for potential endocrine disrupting activity via the ER pathway. This information would be used as one input into a prioritization process for the EPA EDSP. A proposed first tier for EDSP will be to use *in vitro* assays, such as described here, to prioritize chemicals for inclusion in the more complex and costly later tiers of EDSP.[32] For the ~1,800 chemicals evaluated in this study, 82% did not display indications of interacting with the ER signaling pathway and would be low priorities for additional ER testing. If maximum sensitivity is desired, the model can be run with narrower confidence intervals around the composite curves (Figure 3). This would result in an increased false positive rate and a decreased false negative rate.

In summary, an ER Interaction Score was developed by aggregating data from 13 different *in vitro* ER assays based on the known cellular ER signaling pathways. This model produced scores for an overall likelihood of a chemical being estrogenic, and these scores were highly correlated with *in vivo* data and ER reference chemical classifications, indicating that the model is capable of predicting estrogenic likelihood with a high degree of accuracy.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1 illustrates the impact of the linear transformation on the distributions of model R2 values. Figure S2 is the hierarchical clustering of composite scores. Figure S3 is the volcano plot of the observed spectrum of ERα and ERβ activity. Figure S4 is the volcano plot of the ER Interaction Score compared with available uterotrophic data. Figure S5 demonstrates the stability of the CSagonist after removing a single assay from the model. Figure S6 is the hierarchical clustering of all of the in vitro AC50 data. Figure S7 is the comparison of individual assay AC50 data with ER Interaction Scores. Table S1 is the ER reference chemical classifications. File S1 is a PDF of the file containing all the CSbinding plots. File S2 is a CSV file containing all of the output parameters from the CSbinding models. File S3 is a PDF of the file containing all the CSagonist plots. File S4 is a CSV file containing all of the output parameters from the CSagonist models. File S5 is a PDF of the file containing all the CSantagonist plots. File S6 is a CSV file containing all of the output parameters from the CSantagonist models. File S7 is a PDF of the file containing all the CSgrowth plots. File S8 is a CSV file containing all of the output parameters from the CSgrowth models. File S9 is a CSV file containing summaries of the CSbinding, CSagonist, CSantagonist, CSgrowth, and ER Interaction Scores. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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