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Test Driving ToxCast: Endocrine Profiling for 1858 Chemicals Included in Phase II

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Chemical compounds

Bisphenol AF (PubChem CID: 73864); Bisphenol B (PubChem CID: 66166); 2,2',4,4'-Tetrahydroxybenzophenone (PubChem CID: 8571); 2,2',6,6'-Tetrachlorobisphenol A (PubChem CID: 6619); 2,4-Dihydroxybenzophenone (PubChem CID: 8572); Benzyl bromoacetate (PubChem CID: 62576); Cyclohexylphenylketone (PubChem CID: 12837); Bis(2-ethylhexyl) tetrabromophthalate (PubChem CID: 117291); Allura Red C.I.16035 (PubChem CID: 6093299); FD&C Yellow 5 (PubChem CID: 164825)

Conflict of interest

The authors declare they have no competing financial interests with respect to this manuscript, or its content, or subject matter.

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Author contributions

DF, HP, TS, DR, and KT conceived of the scope of the review and contributed to manuscript preparation. DF conducted the analyses of ToxCast data. TS conducted analyses of NIEHS extramural funding portfolios.

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Abstract

Identifying chemicals, beyond those already implicated, to test for potential endocrine disruption is a challenge and high throughput approaches have emerged as a potential tool for this type of screening. This review focused the Environmental Protection Agency's (EPA) ToxCast™ high throughput in vitro screening (HTS) program. Utility for identifying compounds was assessed and reviewed by using it to run the recently expanded chemical library (from 309 compounds to 1858) through the ToxPi™ prioritization scheme for endocrine disruption. The analysis included metabolic and neuroendocrine targets. This investigative approach simultaneously assessed the utility of ToxCast, and helped identify novel chemicals which may have endocrine activity. Results from this exercise suggest the spectrum of environmental chemicals with potential endocrine activity is much broader than indicated, and that some aspects of endocrine disruption are not fully covered in ToxCast.

Keywords

androgen; endocrine disrupting chemicals; endocrine disruptors; estrogen; glucocorticoid; green chemistry; monoamines; noradrenalin; peroxisome proliferator-activated receptor; serotonin; thyroid; toxicology

Introduction

Endocrine disrupting chemicals (EDCs) are exogenous chemicals, or mixture of chemicals, that can interfere with any aspect of hormone action[1]. Many of these substances have been linked with developmental, reproductive, neural, immune, and other problems in wildlife and laboratory animals [2]. Most environmental chemicals have never been fully assessed for potential endocrine activity, thus information regarding how many EDCs exists in our environment, how humans are exposed, and to what degree they pose a risk to human health is limited [2]. As the largest funding agency for EDC research, the National Institute of Environmental Health Sciences (NIEHS) has tended to focus on a relatively small number of chemicals such as bisphenols, phthalates, halogenated persistent compounds, genistein and other isoflavones, and certain pesticides (Figure 1). This focus on a relatively few chemicals is partly due to a lack of research tools to help identify and prioritize which chemicals to study based on indications of potential endocrine activity. Moreover, it can be very difficult to obtain funding support to generate descriptive information on data-poor chemicals in the absence of a specific hypothesis to test. Because chemicals can enter the marketplace (with some exceptions) without any toxicity information, researchers typically have nothing to go on to develop hypotheses.

High throughput screening approaches within the toxicological community hope to help identify chemicals with potential endocrine activity. The USEPA ToxCast program has completed Phase II screening efforts of 1858 unique chemicals in hundreds of HTS assays [3] (Figure 2). Screening programs like ToxCast are a major component of modern toxicology, necessitated by the recognition that animal studies alone are insufficient to generate data on thousands of chemicals in terms of cost, time, and throughput. Previously, Reif et al. [4] developed a Toxicological Priority Index (ToxPi) model to describe and

prioritize 309 unique chemicals for potential endocrine activity using Phase I ToxCast data. ToxPi is an information graphic used to display information on a chemical's bioactivity profiles, inferred toxicity pathways, dose estimates, exposure data, chemical structural descriptors, or other features [5]. The purpose of this article is twofold. First, we use state of the art approaches for analyzing HTS data to identify environmental chemicals with potential endocrine activity. We did this by updating the previous prioritization scheme with Phase II ToxCast data, covering an additional 1549 chemicals. In addition to expanding the chemical coverage, we also made minor changes to the model methodology and included several other EDC assay targets of growing interest in environmental health. The updated prioritization scheme focuses on the classic targets of the estrogen, androgen, and thyroid pathways as well as targets of emerging interest such as the glucocorticoid receptor, peroxisome proliferator-activated receptors (PPARs), and monoamine signaling. Second, through this exercise, we review the strengths and weaknesses of the ToxPi tool, and identify potential new endocrine disruptors which might be an important focus of future research efforts. Thus, this review both summarizes and enhances ToxPi-based screens of ToxCast chemicals for endocrine disrupting chemicals and advocates for engagement and further work on strengthening screening and prioritization strategies.

Publicly Available ToxCast Data

ToxCast currently provides results from 821 assay endpoints that make use of numerous technology platforms from 7 vendors [3] (Figure 2). These platforms include both cell-free (biochemical) and cell-based measures in multiple human primary cells, human or rodent cell lines, and rat primary hepatocytes [6]. A wide spectrum of biological targets or effects is covered, including cytotoxicity, cell growth, genotoxicity, enzymatic activity, receptor binding, reporter gene activity (mostly nuclear receptors), ion channels, and transcription factor activity. Assays were run by the individual vendors and data were provided to EPA for normalization and additional processing [7]. The ToxCast program utilizes novel R scripts to conduct automated curve fitting for each assay–chemical combination to calculate an estimate of potency using a log-transformed AC50 (half-maximal activity concentration). A logAC50 value of 0 is assigned for inactive chemicals. ToxCast also provides an estimate of efficacy, represented by Emax, by taking the maximum of mean response values by concentration. The specific criteria for determining the activity of a compound are technology platform dependent and described elsewhere [3].

The 1858 chemicals tested in ToxCast Phase I and II come from a wide variety of sources including pesticide actives and inert ingredients, industrial and consumer products, potential “green” chemicals that could be safer alternatives to existing chemicals, pharmaceuticals, failed pharmaceuticals, chemicals evaluated in NTP toxicity tests, and chemicals evaluated in the EPA Integrated Risk Information System (IRIS) program (<http://www.epa.gov/ncct/dsstox/>). Importantly, a number of potential and known EDCs have not been tested in ToxCast, including many chlorinated or brominated persistent organic pollutants such as polychlorinated biphenols (PCBs), dioxins/dioxin-like chemicals, and polybrominated diphenyl ethers (PBDEs). Reasons chemicals are not tested in ToxCast, include solubility issues, high volatility, and propensity to bind to plastic.

The US EPA also provides the EPI Suite™ computer program for estimating the physical/chemical properties and environmental fate of chemicals based on chemical structure in SMILES notation [8]. For more information about EPI Suite please refer to: <http://www.epa.gov/opptintr/exposure/pubs/episuite.htm>. In this review of the ToxCast HTS endocrine data we utilize the potency and efficacy estimates provided by the ToxCast program as well as estimates for the LogP (log octanol/water partition coefficient) and BCF (bioconcentration factor using Arnot-Gobas method) as calculated by EPI Suite. ToxCast data files, including information about the chemicals tested, and descriptions are available at <http://epa.gov/ncct/toxcast/data.html>.

Updates to ToxPi Prioritization Scheme

The methodology used in this review builds on previous work by Reif et al. [4] where 309 chemicals tested in Phase 1 of ToxCast were profiled for potential estrogen, androgen, and thyroid disruption. In this review we expand the original prioritization scheme by summarizing the currently available data for the larger 1,858 chemical ToxCast library. In addition to traditional endocrine disruption targets, we highlighted an expanded list of endocrine-related targets to include areas of emerging research interest in environmental health. Specifically, we chose to highlight glucocorticoid and PPARs individually, and added the monoamines noradrenaline and serotonin because of their role as neurotransmitters in the central and peripheral nervous system. The updated prioritization scheme also substituted the estimate of human absorption (Caco-2) used previously for bioconcentration factor (BCF), because both LogP and BCF can be calculated using the freely available EPI Suite program.

We made minor adjustments to the underlying ToxPi calculations to normalize overall ToxPi scores and diminish the influence of cytotoxicity in the prioritization. Previously, overall scores were proportional to the number of slices, making it difficult to compare ToxPi scores across different models. To normalize ToxPi scores we scaled the slice weights, such that the sum of the slice weights is 1. This change constrains all ToxPi scores between 0 and 1, where a score of 1 would indicate the chemical scored highest in every input to the model. While this adjustment will not influence chemical rankings within a given ToxPi model, it facilitates score interpretation across different ToxPi models.

To diminish the effect of cytotoxicity in the ToxPi model, all of the ToxCast results are weighted based on the activity of 27 ToxCast assays identified as good measures of cytotoxicity (the complete list available in Supplemental Materials), hereafter referred to as cytotoxicity assays. For chemicals active in at least 3 cytotoxicity assays, we looked at the distribution of logAC50 values for the active cytotoxicity assays and calculated the cytotoxicity point as the 90th percentile of the distribution. We assigned the cytotoxicity point as 3 (equivalent to 1 mM) for chemicals not active in at least 3 cytotoxicity assays to allow us to calculate a cytotoxicity difference for all chemicals. We chose 1 mM as the default cytotoxicity point because 1 mM fell outside the tested concentration range for every chemical-assay combination, but was not so high as to not add excessive bias for chemicals not active in at least 3 concentrations. For every chemical-assay combination the logAC50

and the Emax were multiplied by the difference between the cytotoxicity point and the logAC50.

For this ToxPi prioritization scheme we considered both potency and efficacy for the specific receptor slices. Efficacy slices are annotated with the postfix “Emax”. We only included efficacy slices for specific receptor endpoints that were assessed in multiple technology platforms. It is important to note that assays covering the same endpoint may use different technologies to characterize the nature of the interaction, e.g., receptor binding or reporter gene. To normalize for the differential dynamic range and normalization (percent of control vs. fold-change) across technology platforms, all efficacy data was transposed onto a standard normal distribution and transformed such that all values were positive. In the models presented here, all slices are equally weighted so that each slice has the same potential contribution to the overall ToxPi score.

Not all chemicals were tested in every technology platform/assay. Missing chemical data is considered inactive in the model. Missing chemical-assay pairs are listed as NA in the input files provided in Supplemental Materials. All analyses were implemented using R [9]. Source code is available upon request. Individual screening results, information about the model slices (including specific assays and the number of unique chemicals tested in each assay), and the model results with the overall ToxPi score and individual slice scores for all 1858 chemicals are available in Supplemental Materials and visually in Figure S1.

Updated EDC ToxPi Findings

Figure 3 summarizes one approach for identifying some of the most active environmental compounds. We show the ToxPi profiles for the 30 top scoring chemicals, excluding pharmaceutical and endogenous compounds. The list includes chemicals with specific profiles, like zearalenone and bisphenol AF showing potential estrogenic activity, and chemicals like apigenin with less specific profiles. We also demonstrate an alternative strategy where we identified the top scoring chemicals for each specific endocrine activity (Figure 4). Again, excluding pharmaceutical and endogenous compounds, we show the top five environmental chemicals along with a known reference chemical for each endocrine activity. Reference EDCs are endogenous or pharmaceutical chemicals often used as positive controls for experimental studies. We selected reference chemicals based on the likelihood of familiarity by researchers in endocrinology. Regardless of which method is used to identify the most active compounds, the results of this analysis suggest the spectrum of environmental chemicals with potential endocrine activity is much broader than “classic” EDCs of current research focus (Figure 1).

There are an estimated 85,000 chemicals in our environment [10], the vast majority of which have not been tested for toxicity [11] and only a few that have been assessed for EDC activity. Moreover, understanding regarding EDC activity is weakened by limited knowledge about modes of action beyond estrogen, androgen, and thyroid hormone interference, and dose response characteristics. For example, that EDCs might promote obesity is still a relatively novel concept and the scope of mechanisms by which this occurs, and over what dose ranges, remain poorly characterized. Using tools like ToxCast can help

prioritize which chemicals may warrant deeper scrutiny in the USEPA's Endocrine Disruptor Screening Program (EDSP) or other research efforts and also help identify novel modes of action. For example the approach shown here identified 141 22 0 (a component of castor oil used as a contraceptive and plasticizer) and N dodecanoyl N methylglycine (a cosmetic agent and surfactant) as being active in the PPAR slice, suggesting they may have metabolic endocrine disrupting effects. Establishing whether or not this is accurate via further testing will simultaneously assess the predictive power of the ToxCast system for this slice and also potentially identify a novel EDC of concern and its mode of action. Similarly, use of ToxCast, as we have shown here, also has the potential to identify emerging contaminants with endocrine disrupting properties before they become pervasive. For example, the bisphenol A (BPA) analogues bisphenol AF and bisphenol B were among the more endocrine active environmental chemicals tested in Phase II of ToxCast (Figure 3), and corroborated by other recent findings [12]. Use of these analogues may increase as businesses strive to find alternatives to BPA in products. The type of data generated from ToxCast can provide valuable information both to chemists developing new chemicals as well as toxicologists working to design toxicity studies for chemicals of emerging concern.

The next steps in considering results from ToxCast data should include confirming the presented results with similar *in vitro* methods. All source assay contracts include specific performance criteria for delivered data, and extensive chemical QC efforts [13] that track substances back to the sample origin aim to minimize artifacts introduced by chemical irregularities. Nonetheless, across such a diverse chemical space, there will be variability. To address this, the assay set was designed with partial redundancy in mind. Although every possible pathway of toxicological interest is not covered evenly, the partial overlap in assay coverage and high dimensionality of the endpoint suite render results more robust when used in an ensemble manner, as with ToxPi. There is greater confidence in the screening results when chemicals show activity across orthogonal assays targeting the same gene or biological process. For example, the presented model includes 21 assays across multiple technology platforms, both cell-free and *in vitro*, for assessing potential estrogenicity. The estrogen-related assays cover multiple aspects of the estrogen signaling pathway, including estrogen receptor ligand binding, receptor dimerization, gene transcription, and estrogen-induced cell proliferation. Conversely, the monoamine slice only contains 13 cell-free ligand binding assays. Notably, as we conceptualized and developed the ToxPi slices for these analyses, there was interest in assessing other neural gene targets known to be influenced by EDCs including oxytocin and vasopressin but assay coverage was concluded to be inadequate to ensure confidence in the results. Identifying these types of limitations will ultimately be useful and important when considering improvements to enhance coverage and utility. Furthermore, these *in vitro* screening results only address potential hazard. We explicitly do not address exposure or pharmacokinetics, but understand the necessity of considering these factors in evaluating risk to human health. As more researchers take advantage of the growing ToxCast database, and employ the ToxPi tool with different parameters and slices designed to capture different aspects of endocrine activity, dialog about these types of limitations, and how to address them, will be advantageous and provide avenues for enhancement of the screening program.

Conclusions

We encourage others to use the public ToxCast data to explore their own hypotheses about specific axes of toxicological hazard probed by this diverse assay set. Here, we have explored one such axis, endocrine activity, by building a ToxPi prioritization scheme from relevant ToxCast assays and placing the results in context with reference chemicals having known EDC activity. Information on the assays can be accessed through the iCSS Dashboards (<http://actor.epa.gov/dashboard/>), which includes browsing capabilities as well as links to download entire data sets. A graphical user interface (GUI) is available for ToxPi that provides a user-friendly way to (re)combine data from ToxCast and/or any other source [5]. This framework has been used for meta-analysis of studies across labs, exploration of cardiotoxicity endpoints from induced pluripotent stem cells, and integration of chemistry-based structure-activity relationship models (SAR) with biological assay results [14–16]. The utility of such a large chemical screening effort—where each chemical has full-rank assay data—will be realized when researchers examine focused screening results within the broad context of ToxCast and related screening and testing efforts.

One goal of this updated analysis and review was to identify and highlight what might be novel EDCs of interest for further research. There is no question that a select group of chemicals have played a large and cardinal role in advancing EDC research, but the novel chemical identified herein highlight the need for diversification. A brief analysis of the projects funded through the NIEHS Division of Extramural Research and Training (DERT) starting before January 2014 and remain active indicates certain EDCs --for example, phthalates, and BPA -- continue to be heavily studied in DERT scientific portfolios (Figure 1). Of 934 active research projects in the portfolio, 210 address one or more of these specified target chemicals. About 18% of the DERT budget goes toward research on these EDCs, the majority of that on BPA. These few chemicals may maintain prominence simply because they have been well researched in the past, not because they pose the most pressing risk to human health. This effect, known as the Matthews principle [17], may represent a self-serving bias in EDC research, in which renewal and enhancement of funding, high citation rates, and attention within specialized scientific groups (and the media) perpetuates focus on a small subset of chemicals. As new chemicals and modes of action are identified in screening platforms such as ToxCast, researchers and funders may benefit from diversifying their goals by casting a wider net that includes less well-known chemicals. Going forward we advocate for strengthening screening platforms, validating the activity of compounds identified as potential endocrine disruptors in those assays, and diversifying funding and research efforts to better characterize the potential health risks of EDC exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- 1858 chemicals in EPA's ToxCast program were analyzed for endocrine assay targets.
- ToxPi model utilizes 85 *in vitro* HTS assays and 2 physicochemical properties.
- We focused on estrogen, androgen, thyroid, glucocorticoid, PPARs and monoamines.
- Many chemicals showing activity in the *in vitro* assays have not been studied for potential endocrine disruption.
- Additional research is required to confirm these screening level analyses.

Figure 1. Chemical-Health Outcomes Combinations for Top-Studied EDCs

The research projects included in these charts started before January 2014 and remain active (individual grants or subprojects of multi-project grants) in the NIEHS portfolio. 460 Chemical-outcome associations were determined based on co-occurrence within individual projects. Exposures not studied in the context of any outcomes are not represented in these charts. Skin, microbiome, and musculoskeletal tags are grouped and added to the “Other Outcomes” category.

*Chemical not tested in ToxCast Phase II.

Abbreviations: BPA = bisphenol A; PCBs = polychlorinated biphenyls; PBDEs = polybrominated diphenyl ethers; PFC = perfluorocarbons; PFOA = perfluorooctanoic acid; PFOS = perfluorooctane sulfonate; TCDD = 2,3,7,8-Tetrachlorodibenzo-p-dioxin (“dioxin”); DDT/DDE = dichlorodiphenyltrichloroethane/dichlorodiphenyldichloroethylene; Gen = genistein

Figure 2. Schematic of ToxCast results recombined into a ToxPi model

Proceeding about the figure in a clockwise manner from the top left: The activity of each chemical ($N = 1858$) on each assay is summarized by an AC50 (actives) or null (inactives). The activity calls are assembled into a results matrix, where rows contain information for each chemical and columns contain information for each assay. In the conceptual representation of the data matrix shown here, the width of each shaded portion is proportional to the number of assays from each source technology platform. For the ToxPi model described here, a subset of relevant assays is selected from the full results matrix, shown here as a matrix with the same number of chemical rows (N) and fewer assays (columns). These assays are then recombined into a ToxPi model, where assays of different types have been organized into slices of biological relevance.

Figure 3. Screening for identifying potential EDCs based on overall model score

An approach to identifying potential EDCs focusing on the overall model score. A: The ToxPi for the highest scoring 30 chemicals without a pharmaceutical use-case in the iCSS Dashboard, plotted in decreasing order by their respective ToxPi model score. Each ToxPi represents one chemical, with the chemical name below the ToxPi and overall model score in parentheses. The model also included the physical properties bioconcentration factor (BCF) and partition-coefficient (log P) to help in prioritization. B: The distribution of ToxPi scores for all 1858 chemicals in the model. Blue points highlight the location of the signpost chemicals listed on the y-axis, and the red points highlight the chemicals listed in A relative to the whole chemical library. Abbreviations: HPTE = 2,2-bis(p-hydroxyphenyl)- 1,1,1-trichloroethane; *p,p'*-DDD = *p,p'*-Dichlorodiphenyl dichloroethane; 141-22-0 = CAS registration number for 9-Octadecenoic acid, 12-hydroxy-, (9Z,12R)-

Figure 4. Screening for identifying potential EDCs based on specific endocrine pathway
An approach to identifying potential EDCs focusing on specific endocrine pathways. A: Each grouping of ToxPi represent one of the endocrine axes identified in the model. For each endocrine axis we show one reference compound and the top scoring five chemicals, without a pharmaceutical use-case in the iCSS Dashboard, for the respective endocrine axis. Each ToxPi represents one chemical, with the chemical name below the ToxPi and overall model score in parentheses. The model also included the physical properties bioconcentration factor (BCF) and partition-coefficient (log P) to help in prioritization. B: The distribution of ToxPi scores for all 1858 chemicals in the model. Blue points highlight the location of the signpost chemicals listed on the y-axis, and the red points highlight the chemicals listed in A relative to the whole chemical library.
Abbreviations: HPTE = 2,2-bis(p-hydroxyphenyl)- 1,1,1-trichloroethane; 141-22-0 = CAS registration number for 9-Octadecenoic acid, 12-hydroxy-, (9Z,12R)-; 26040-51-7 = CAS registration number for Bis(2-ethylhexyl) tetrabromophthalate