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Endocrine Disruptors: Data-based survey of *in vivo* tests, predictive models and the Adverse Outcome Pathway

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ABSTRACT

The protection from endocrine disruptors is a high regulatory priority. Key issues are the characterization of *in vivo* assays, and the identification of reference chemicals to validate alternative methods. In this exploration, publicly available databases for *in vivo* assays for endocrine disruption were collected and compared: Rodent Uterotrophic, Rodent Repeated Dose 28-day Oral Toxicity, 21-Day Fish, and *Daphnia magna* reproduction assays. Only the Uterotrophic and 21-Day Fish assays results correlated with each other. The *in vivo* assays data were viewed in relation to the Adverse Outcome Pathway, using as a probe 18 ToxCast *in vitro* assays for the ER pathway. These are the same data at the basis of the EPA agonist ToxERscore model, whose good predictivity was confirmed. The multivariate comparison of the *in vitro*/*in vivo* assays suggests that the interaction with receptors is a major determinant of *in vivo* results, and is the critical basis for building predictive computational models. In agreement with the above, this work also shows that it is possible to build predictive models for the Uterotrophic and 21-Day Fish assays using a limited selection of Toxcast assays.

1. Introduction

Increasing incidence of disorders such as obesity/diabetes/metabolic syndrome, reproductive dysfunction, and neuro-developmental abnormalities in some human populations have raised concern that disruption of key endocrine-signaling pathways by exposure to environmental chemicals may be involved. The endocrine system consists of an assemblage of ductless glands that secrete hormones directly into the blood or lymph, which regulate a wealth of biological processes. The endocrine system is comprised of multiple pathways, or axes, each consisting of different groupings of organs and hormones with distinct regulatory functions. These pathways are intricately involved in organizational, or programming, events during fetal development, as well as in the maintenance of homeostasis in the adult organism. Evidence to date indicates that hormone nuclear receptors are a major target of endocrine disrupting chemicals (EDCs) because these receptors are designed to bind small, lipoidal molecules (i.e., steroid hormones), which can be mimicked by many environmental chemicals. These nuclear receptors, once activated by their ligand, regulate the transcription of target genes, whose products —in turn-initiate a cascade of events eventually leading to organismic effects. To cope with such concerns, the protection of human health and of the environment from endocrine disruptors has become a high priority for regulatory authorities, that are considering a large variety of approaches (OECD, 2012; Solecki et al., 2017).

A Conceptual Framework (CF) for the Testing and Assessment of Endocrine Disruptors was adopted by the Organization for the Economic Co-operation and Development (OECD) in 2002. The CF is not a testing strategy; it is not prescriptive and simply reflects the type of information the tests provide at the different levels, such as informing endocrine toxicity outcome pathways, moving from *in silico* to *in vitro* and *in vivo*. It should be emphasized that information on mechanisms/pathways is considered particularly important for assessing the EDCs. An important component of the OECD initiative are a series of Guidance Documents, and Testing Guidelines (TG) on established assays. Methods are aimed at both the mechanistic evaluation of the action of EDCs, and the assessment of their physiological consequences. Detailed information is available in the OECD website: http://www.oecd.org/env/ehs/testing/oecdworkrelatedtoendocrinedisrupters.htm.

To determine the usefulness and limitations of a novel

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alternative method for identifying endocrine activity, the method must be evaluated against a set of chemicals that have demonstrated activity and well-defined properties *versus* the target nuclear receptor and subsequent biological pathway. However -because of the limited amount of available *in vivo* data-most often reference chemicals are selected based only on their activity in other *in vitro* assays, in a circular validation paradigm (OECD, 2012; Browne et al., 2015). Thus, the validation of methods is a key issue for the progress of the science in this field.

In this field, new initiatives are continuously arising. For example –and of particular importance-within the ToxCast program the U.S. Environmental Protection Agency (EPA) has implemented 18 *in vitro* High-Throughput (HTS) assays that probe the Estrogen Receptor (ER) pathway in mammalian systems. Results from the 18 screening assays were integrated into a computational model that can discriminate bioactivity from assay-specific interference and cytotoxicity. EPA is accepting ToxCast ER model data for 1812 assayed chemicals as alternatives for EDSP Tier 1 ER binding, ER Transactivation, and Uterotrophic assays (Browne et al., 2015). In addition, the EPA has curated and made publicly available a database of Uterotrophic assay results, which can be considered the "gold standard" to identify potential ER agonists (Kleinstreuer et al., 2016).

The present work is an exploratory analysis aimed at contributing to this validation. It compares results from a series of *in vivo* assays indicated by OECD as indicators of potential endocrine disruption. In addition, it puts results in the context of the Adverse Outcome Pathway (AOP) conceptual framework for the EDCs, and suggests implications for further development of predictive methods.

2. Data and methods

2.1. In vivo assays for EDCs: databases of results

The *in vivo* toxicity assays indicated by the OECD as suitable for screening potential EDCs, as listed in: http://www.oecd.org/env/ehs/testing/oecdworkrelatedtoendocrinedisrupters.htm, are:

- -Uterotrophic Bioassay in rodents (a short-term Screening Assay for Oestrogenic Properties)
- -Repeated Dose 28-day Oral Toxicity Study in Rodents
- -*Daphnia magna* Reproduction Test
- -21-Day Fish Assay (a Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition).

The Uterotrophic assay data were retrieved from the Supplemental Material to the paper (Kleinstreuer et al., 2016).

Data relative to the other *in vivo* endpoints were retrieved from databases contained in the OECD QSAR Toolbox (http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm) (Van Leeuwen et al., 2009; Benigni et al., 2012; Dimitrov et al., 2016).

For the *Daphnia magna* results, Toolbox has two different compilations (Ecotox and Aquatic Japan MOE). They were maintained separate for this analysis.

For the Repeated Dose assay, the Toolbox has two compilations, but only one (Repeated Dose Toxicity HESS) is freely downloadable. The data were downloaded by filtering for compliance to the OECD Testing Guideline 407, and limitedly to the endpoints considered to be relevant to endocrine effects, i.e., adrenal; epididymis; ovary; prostate; testis; thyroid; uterus (see Annex 2 of the OECD TG 407: http://www.oecd-ilibrary.org/environment/test-no-407-repeated-dose-28-day-oral-toxicity-study-in-rodents_9789264070684-en).

In addition to the above databases, the Toolbox includes two databases of chemicals reviewed and classified by experts from Procter and Gamble for Reproductive and Developmental effects. This is part of the DART predictive system implemented in the Toolbox, according to (Wu et al., 2013). The database was compiled from a range of different sources, including previous databases as well as primary literature. In this paper, the DART database for Reproductive effects was considered in the analyses.

Preliminarily to the analyses, to ensure homogeneity all data were transformed into negative/positive calls. For the Uterotrophic assay in case of reported contradictory studies results, the positives were those with a majority of positive studies. For the Repeated Dose endpoint, the chemicals with a reported LOEL were considered as positive, whereas those with only reported NOEL were considered as negative. For the 21-Day Fish Assay, the chemicals with reported LOEL or LOEC were considered as positive, whereas those with only reported NOEC or NOEL were considered as negative. For the *Daphnia magna* Reproduction Test, the chemicals with EC50 were considered as positive and those only with reported NOEC were considered as negative.

The numbers of chemicals in the databases are the following (Table 1):

Daphnia magna Reproduction Test, Aquatic Japan MOE database, n = 352.

Daphnia magna Reproduction Test, Ecotox database, n = 392.

DART database for Reproductive effects, n = 289.

Repeated Dose 28-day Oral Toxicity Study in Rodents, n = 178.

Uterotrophic Bioassay in rodents, n = 114.

21-Day Fish Assay, n = 44.

2.2. High-throughput data for ER bioactivity

In the EPA's ToxCast program, potential ER bioactivity was measured in 18 HTS *in vitro* assays. The suite of HTS assays measure the molecular initiating event (i.e., receptor binding), in addition to several key events (e.g., receptor dimerization, DNA binding, transactivation, gene expression, and cell proliferation) in an AOP.

The 18 ER assays include three cell-free biochemical radioligand ER binding assays; three protein complementation assays that measure formation of ER dimers and test for activity against ERα and ERβ, each measured at two time points; an assay measuring interaction of the mature transcription factor with DNA at two time points; two reporter gene assays measuring RNA transcript levels; two assays measuring reporter protein levels; an ER-sensitive cell proliferation assay; and two transactivation antagonist assays (Browne et al., 2015; Judson et al., 2015).

All of the raw and processed data, as well as annotations are publicly available: http://epa.gov/ncct/toxcast/data.html and http://actor.epa.gov/edsp21.

We used a computation-ready file of data generously provided by Nicole Kleinstreuer.

2.3. ToxERscore predictive model

The U.S. Environmental Protection Agency (EPA) has integrated 18 *in vitro* HTS assays into a computational model that can discriminate bioactivity from assay-specific interference and cytotoxicity. The Agonist AUC value of the ToxERscore model is described in detail in (Browne et al., 2015; Judson et al., 2015). Scores calculated with the model for individual 1812 chemicals were downloaded from the Supplemental material of the (Browne et al., 2015) paper.

The data used in the analyses (*in vivo* data, and the Toxcast High-throughput data for ER bioactivity) are provided in the Supplemental Material.