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An exposure:activity profiling method for interpreting high-throughput screening data for estrogenic activity—Proof of concept

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ABSTRACT

Rapid high throughput *in vitro* screening (HTS) assays are now available for characterizing dose–responses in assays that have been selected for their sensitivity in detecting estrogen-related endpoints. For example, EPA's ToxCast™ program recently released endocrine assay results for more than 1800 substances and the interagency Tox21 consortium is in the process of releasing data for approximately 10,000 chemicals. But such activity measurements alone fall short for the purposes of priority setting or screening because the relevant exposure context is not considered. Here, we extend the method of exposure:activity profiling by calculating the exposure:activity ratios (EARs) using human exposure estimates and AC50 values for a range of chemicals tested in a suite of seven estrogenic assays in ToxCast™ and Tox21. To provide additional context, relative estrogenic exposure:activity quotients (REEAQ) were derived by comparing chemical-specific EARs to the EAR of the ubiquitous dietary phytoestrogen, genistein (GEN). Although the activity of a substance in HTS-endocrine assays is not a measure of health hazard or risk, understanding how such a dose compares to human exposures provides a valuable additional metric that can be used in decision-making; substances with small EARs and REEAQs would indicate low priority for further endocrine screening or testing.

1. Introduction

The intent to reduce, refine, and in some instances, replace the use of animals in toxicity testing has spurred the development of high-throughput screening (HTS) assays that eventually may be used for chemical risk assessment (Judson et al., 2011, 2014; Thomas et al., 2013). However, to date, many of these *in vitro* approaches have demonstrated limited applicability for predicting *in vivo* hazard using statistical classification methods (Thomas et al., 2012). Initially, it was proposed that the use of classification-based prediction models derived from HTS assays results were capable of matching the *in vivo* estrogen and androgen results of the Endocrine Disruptor Screening Program Tier 1 battery with high balanced accuracies approaching 100%, with notable data gaps for thyroid and steroidogenesis pathways (Rotroff et al., 2013a, 2014; Cox et al., 2014). However, when predictive models were developed from the Rotroff et al. (2013a) dataset using a cross-validation approach, the balanced accuracies for predicting *in vivo* endpoints fell to 85% for estrogen and 79% for androgen pathways (Cox et al., 2014). Recently, an approach that combines results from different estrogen pathway-based HTS assays into an ER Interaction Score has been suggested as a potential tool for prioritizing chemicals for further endocrine screening (Rotroff et al., 2014). Using a limited set of known positives and known negatives, the ER Interactions Score methodology was shown to have 91% sensitivity and 65% specificity (*Ibid.*). It is evident from these early efforts to prioritize chemicals for further estrogen pathway-related

Abbreviations: HTS, high throughput screening; EDSP, EPA's Endocrine Disruptor Screening Program; ER, estrogen receptor; EAR, exposure:activity ratio; AC50, concentration at half maximal activity; REEAQ, relative endocrine exposure:activity quotients; GEN, genistein; BMD, bench mark dose; BPA, Bisphenol-A.

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effects that more exposure context needs to be provided to permit a transparent, science-based approach for identifying priority chemicals. There are even larger barriers, including a lack of appropriate HTS assays, for the use of HTS data to predict and prioritize substances for potential activity in thyroid and steroidogenesis pathways, (Rotroff et al., 2013a, Paul et al., 2014). Despite these limitations, such HTS methods hold great promise for improving the biological basis for priority setting and chemical screening, and may critically support targeted testing with a tiered, risk-based framework (Thomas et al., 2013; Pastoor et al., 2014).

While application of HTS results to predict adverse effects for use in risk assessment may be futuristic, scientific support is growing for more immediate applications in priority setting, particularly for endocrine screening, such as within EPA's tiered testing and assessment approach, the Endocrine Disruptor Screening Program (EDSP). EPA has articulated its projected use of HTS methods in its "EDSP21" vision, which projects utilization of HTS approaches initially for priority setting, and then as experience is gained, and commensurate with achieving the requisite degree of scientific confidence, as replacements for specific EDSP screens and tests (USEPA, 2011, 2014b). Early efforts have been made to characterize the prediction landscape provided by HTS assay data from the ToxCast™ and Tox21 programs; these efforts were aimed at providing methods for ranking chemicals by various metrics. Reif et al. (2010) presented results of ToxCast™ endocrine screening using the ToxPi visualization and ranking tool. This endocrine ToxPi assembled the results of related assays into specific sections of a pie chart, and then for each compound, the area reflected its activity in a specific set of related ToxCast™ assays, with larger areas suggesting increasing hazard and/or potency. However, ToxPi does not employ standard reference compounds for normalizing responses, and instead normalizes each analysis in relation to the compound with the highest level of activity among the set of substances evaluated (Patlewicz et al., 2013). This relativistic approach falls short by not providing potencies benchmarked to reference substances, and therefore the predicted responses cannot be prioritized within the context of a well-characterized biological effect. In the absence of this context for priority setting using endocrine-related HTS data, the point at which a potential biological effect falls below a regulatory level of concern becomes unclear, and the relative priorities may even be inconsistent with the large database of available toxicology data from animal studies. The strengths of the recent ER Interaction Score method of Rotroff et al. (2014) include incorporation of data from 13 assays indicative of ER signaling, but a primary weakness is that it uses an approach which compresses the relative potency information for screened chemicals, thereby limiting the utility of this method for priority setting based on integrated consideration of relative potency, dosimetry, and exposure information. The USEPA presented the integrated bioactivity and exposure ranking (IBER) in December 2014 (USEPA, 2014b), a ratio based on a model score similar to the ER Interaction Score and predicted human exposure, for priority-setting; implementation of this approach will likely require determination of IBER values that fall below a level of concern for further testing. The need to consider exposure information along with measures of biologic effects in the initial stages of a risk assessment was articulated in the RISK21 roadmap (Pastoor et al., 2014). Using an approach such as the RISK21 roadmap that is problem formulation based, exposure driven, and expresses the intersection of exposure and effect is an effective means for prioritizing chemicals for further assessment (Pastoor et al., 2014).

Thus, we propose a profiling approach that incorporates standardization to a reference chemical such that the level of concern is rooted in transparent methodology and becomes obvious and evident. This proposed exposure:activity profiling approach reflects standardization tools used historically in toxicology, such as dioxin toxicity equivalence factors and environmental estrogen equivalents (Giesy et al., 2002; Van den Berg et al., 1998, 2006), but goes beyond toxicity equivalence by incorporating exposure information rather than relying solely on a relative measure of potency or toxicity. Accordingly, the priority status or score of a chemical would reflect both exposure and potency (Borgert et al., 2012, 2013). This score would be presented relative to that of a chosen reference chemical to provide appropriate context. In summary, the exposure:activity profiling approach we demonstrate herein supports the use of HTS data in prioritization tasks.

2. Exposure:activity profiling

Here, as a proof of concept, we illustrate how to apply the method of exposure:activity profiling (Becker et al., 2014) to HTS endocrine results by calculating exposure:activity ratios (EARs). This method is a variation of that presented previously (Wetmore et al., 2012, 2013). To demonstrate this method, we selected a set of results from seven HTS assays for estrogen receptor interaction in the ToxCast™ and Tox21 assay battery. Because this is a proof of concept exercise, substances were chosen based on the availability of (1) estimates of current human oral exposures and (2) calculated human oral equivalent doses corresponding to *in vitro* HTS AC50 values (these values were obtained from Supplemental Table 8 of Wetmore et al., 2012). Thus, EARs were developed using human exposure estimates expressed as oral doses in mg/kg/d in comparison to predicted oral equivalent doses, also in mg/kg/d, corresponding to *in vitro* activity in ToxCast™ assays (e.g., AC50 or LEC50 values) (Wetmore et al., 2012). If a chemical was negative in an assay the EAR was defaulted to zero. This approach enabled us to readily calculate EARs without having to independently derive oral equivalent values. The operation and source of the assays is described in detail elsewhere (Judson et al., 2010; Rotroff et al., 2013a,b, 2014), as are descriptions of the publicly available chemical library and quality control measures (Sipes et al., 2013; http://www.epa.gov/ncct/dsstox/). The derivation of assay AC50 values is reported on the ToxCast website (http://epa.gov/ncct/toxcast/data.html, December 2013 release). The *in vitro* HTS bioactivity data were not corrected using a cytotoxicity filter and reflect the publicly-available data from ToxCast™ Phase II at the time the database was accessed.

In enterocytes, in liver, and in other tissues *in vivo* many chemicals with demonstrated estrogenic activity *in vitro* are inactivated by conjugation to the glucuronide or sulfate (Strassburg et al., 2002; Guillemette et al., 2004). Hence, the steady state concentrations in plasma used to determine the EAR will be the bioactive aglycone rather than the total that includes conjugated forms. When using *in vitro* data as the source of activity concentrations, the exposure estimate must be represented as either steady state plasma concentrations derived from blood or urinary concentrations, or from oral equivalent doses (see SF-1 in Supplemental Information for a schematic diagram of the three dosimetric levels—oral equivalents or external dose, steady state plasma concentrations, and urinary excretion values). Because the major portion of GEN, as well as that of many other estrogenic chemicals, exists in plasma as an inactive conjugate, it is necessary to express both the exposure and activity levels as steady state plasma levels of the active moiety. This approach also requires that urinary excretion values of the chemical of interest be expressed as steady state plasma levels of the bioactive aglycone.

For estimating activity values from *in vivo* animal data, Becker et al. (2014) used a BMD value from the uterotrophic assay to obtain the corresponding biomonitoring equivalent (BE) value; thus, they expressed an external dose as a urinary concentration. This enabled comparison of the human exposure values (mg/L in