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Screening the ToxCast Phase 1, Phase 2, and e1k Chemical Libraries for Inhibitors of Iodothyronine Deiodinases

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Abstract

Deiodinase enzymes play an essential role in converting thyroid hormones between active and inactive forms by deiodinating the pro-hormone thyroxine (T4) to the active hormone triiodothyronine (T3) and modifying T4 and T3 to inactive forms. Chemical inhibition of deiodinase activity has been identified as an important endpoint to include in screening chemicals for thyroid hormone disruption. To address the lack of data regarding chemicals that inhibit the deiodinase enzymes, we developed robust *in vitro* assays that utilized human deiodinase types 1, 2, and 3 and screened over 1,800 unique chemicals from the U.S. EPA's ToxCast phase 1_v2, phase 2, and e1k libraries. Initial testing at a single concentration identified 411 putative deiodinase inhibitors that produced inhibition of 20% or greater in at least one of the three deiodinase assays, including chemicals that have not previously been shown to inhibit deiodinases. Of these, 228 chemicals produced enzyme inhibition of 50% or greater; these chemicals were further tested in concentration-response to determine relative potency. Comparisons across these deiodinase assays identified 81 chemicals that produced selective inhibition, with 50% inhibition or greater of only one of the deiodinases. This set of three deiodinase inhibition assays provides a significant

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contribution towards expanding the limited number of *in vitro* assays used to identify chemicals with the potential to interfere with thyroid hormone homeostasis. Additionally, these results set the groundwork for development and evaluation of structure-activity relationships for deiodinase inhibition, and inform targeted selection of chemicals for further testing to identify adverse outcomes of deiodinase inhibition.

Keywords

thyroid; screening; deiodinase; prioritization; endocrine disruption

Introduction

There is increasing evidence that environmental contaminants have thyroid-disrupting properties (Boas et al., 2012; Brucker-Davis, 1998; Duntas and Stathatos, 2015; Zoeller, 2005). Chemical interference with thyroid hormone (TH) homeostasis is of concern because of the essential role of THs in vertebrate development and metabolic processes (Zoeller et al., 2007). Despite the growing number of studies, information on thyroid disruption is still limited to a small number of the thousands of chemicals that need to be assessed for human and ecological effects (U.S. EPA, 2012, 2014, 2017a). To address this data gap, *in vitro* and *in silico* methods must be used to identify potential thyroid-disrupting chemicals (Murk et al., 2013; OECD, 2014).

Thyroid hormone homeostasis is controlled by a complex series of coordinated events dependent on multiple proteins for TH synthesis, transport, and peripheral metabolism and elimination (Brix et al., 2011; Zoeller et al., 2007). Environmental chemicals can perturb this complex system through a variety of mechanisms that result in thyroid disruption (Boas et al., 2012). Until recently, *in vitro* screening assays had been implemented for only a few of the potential molecular targets for thyroid disruption, and the only thyroid-relevant assays included in the U.S. EPA's Toxicity Forecaster (ToxCast) program were the receptor transactivation assays and thyrotropin releasing hormone assays (U.S. EPA, 2015). Recent efforts have identified and described priority targets to use in screening assays to detect potential thyroid-disrupting chemicals (Murk et al., 2013; OECD, 2014). Progress in this area includes the development of *in vitro* screening assays for identifying chemical disruptors of TH synthesis via inhibition of thyroid peroxidase (TPO; Paul et al., 2014) and the sodium-iodide symporter (NIS; Hallinger et al., 2017; Lecat-Guillet et al., 2007), interruption of TH transport (Dong and Wade, 2017; Jayarama-Naidu et al., 2015), and TH activation/inactivation via inhibition of the iodothyronine deiodinases (Hornung et al., 2018; Renko et al., 2012, 2015), with recent screening of chemical libraries for inhibition of TPO (Paul Friedman et al., 2016), NIS (Wang et al., 2018), and one deiodinase isoform (Hornung et al., 2018).

The aim of this study was to screen environmental chemicals for inhibition of the three iodothyronine deiodinase enzymes: deiodinase type 1 (DIO1), deiodinase type 2 (DIO2), and deiodinase type 3 (DIO3). These three distinct deiodinase enzymes are essential in mediating TH action in organs and tissues where they each perform different roles in

converting THs between active and inactive forms, with differences in substrate specificities and tissue-specific expression (Gereben et al., 2008; Köhrle, 1999). DIO2 is important for converting the pro-hormone thyroxine (T4) to the more active hormone triiodothyronine (T3) through the removal of the 5' outer ring iodine. Whereas DIO3 inactivates both T4 and T3 by removing an inner ring iodine, producing reverse T3 (rT3) and diiodotyrosine (T2), respectively. DIO1 targets both the outer and inner rings, and thus can convert T4 to T3 or inactivate either of these THs. These key roles of deiodinases in modulating tissue- and timing-specific levels of T3 and T4 are well-studied, and there is a wealth of knowledge on enzymatic activity and function, tissue and substrate specificity, and relative importance across multiple vertebrates (see reviews: Darras and Van Herck, 2012; Gereben et al., 2008; Köhrle, 1999; Kuiper et al., 2005; Orozco et al., 2012). Although adverse health or developmental effects of chemical inhibition of deiodinases are not well understood, some known thyroid-disrupting compounds may be acting through this pathway (eg, polybrominated diphenyl ethers, Roberts et al., 2015). In addition, altered deiodinase expression has been documented in several types of cancer (Casula and Bianco, 2012) and mammalian knock-out studies demonstrate negative consequences from deiodinase deficiency (Hernandez et al., 2006; Marsili et al., 2011). Chemical inhibition of deiodinase activity has been identified as an important endpoint to include in screening chemicals for TH disruption (Murk et al., 2013; Zoeller et al., 2007). Presently, there is a lack of data regarding the potential of chemicals to inhibit each of the three deiodinase enzymes with few chemicals tested in existing assays (Renko et al., 2015; Schweizer and Steegborn, 2015), apart from recent screening for inhibition of DIO1 (Hornung et al., 2018).

Presented here are development of screening assays for DIO2 and DIO3 and the results for the DIO1, DIO2, and DIO3 inhibition assays from screening over 1,800 chemicals from the ToxCast phase 1_v2 (ph1v2), phase 2 (ph2), and e1k chemical libraries (Richard et al., 2016). First, we screened the ToxCast ph2 and e1k libraries for DIO1 inhibition using the screening assay and approach in Hornung et al. (2018). We then established screening assays for DIO2 and DIO3 through adaptation of the previously reported assays (Hornung et al., 2018; Renko et al., 2015). These assays were used to screen ToxCast ph1v2, ph2, and e1k libraries for DIO2 and DIO3 inhibition using a tiered screening approach with an initial single high concentration (target of 200 μM) of each chemical followed by further concentration-response testing, similar to that used for DIO1 (Hornung et al., 2018) and other thyroid targets (Paul Friedman et al., 2016; Wang et al., 2018). This study significantly expands the current knowledge of chemicals that could disrupt the thyroid axis via inhibition of iodothyronine deiodinase activity and allows comparisons of similarities and differences in chemical inhibitors not previously possible.

Materials and Methods

The development of these deiodinases assays and the chemical screening followed the approach described in Hornung et al. (2018), with adenoviral expression of human deiodinase enzyme, a colorimetric assay that measured the release of iodide from the hormone substrate, and a tiered screening approach with initial testing at a single high concentration followed by a subset of chemicals tested in concentration-response. The Supplementary Data file includes details of the conditions for each assay (Supplementary

Table 1) and the assay plate layouts (Supplementary Figs. 1 and 2) as well as results from two test plates used in development of the DIO2 and DIO3 assays (assay performance metrics in Supplementary Table 2 and inhibition produced by each chemical in Supplementary Tables 3 and 4).

Chemicals

A test set of 1,851 unique chemicals from the ToxCast ph1v2, ph2, and e1k chemical libraries (Richard et al. 2016) was obtained through Dr. Ann Richard (U.S. EPA, Research Triangle Park, North Carolina). These test chemicals were supplied with chemical identities masked in a 96-well plate format at a target concentration of 20 mM in dimethyl sulfoxide (DMSO) with one chemical per well. The actual plated concentration of some chemicals differed from the target of 20 mM due to solubility limitations in DMSO or for oils and mixtures with concentrations provided in mg/ml. As described below under *Assay quality/ performance*, 32 chemicals had evidence of assay interference (see Supplementary Table 5). Thus, the final test set was 1,819 unique chemicals, which are listed with maximum concentration tested in Supplementary Table 6, ordered by ToxCast chemical library and Chemical Abstracts Service Registry Numbers (CASRNs).

Each assay plate included a model inhibitor as a positive control with a complete inhibition curve. For the DIO1 assay, 6-propyl-2-thiouracil (PTU) was used as the model inhibitor, following Hornung et al. (2018) and Renko et al. (2012). For the DIO2 and DIO3 assays, xanthohumol (XTH) was selected for the positive control based on the identification of this chemical as a potent inhibitor of all three deiodinases by Renko et al. (2015). DMSO was the solvent control in all three assays and was considered a negative control that reflected maximum deiodinase activity. The model inhibitors were solubilized in DMSO with graded concentrations to produce an inhibition concentration-response curve (CASRNs and concentrations included in Table 1). To measure intra-assay reproducibility, a small subset of chemicals was replicated across chemical source plates used for the single-point screening. As no standardized set of chemicals exists for deiodinase inhibition, these chemicals were selected based on results in the TPO screening assay (Paul Friedman et al., 2016), with ten ToxCast ph1v2 chemicals in the DIO1 assay and nine of these same chemicals in the DIO2 and DIO3 assays (see Supplementary Figure 3). These chemicals were distributed on the chemical source plates by the supplier with identities masked, with one to six of these chemicals per plate. Chemical plates were sealed and kept at −80 °C when not in use for assays. Chemical reagents, other than the chemical test set, were purchased from Sigma-Aldrich (St. Louis, Missouri).

Adenoviral Expression of Human Deiodinases

Adenoviral expression of the human deiodinases followed the methods previously described for DIO1 (Hornung et al., 2018). A summary and specifics for expression of DIO2 and DIO3 are included below. Human deiodinase expression plasmids for DIO2 (hD2D10; Buettner et al., 2000) and DIO3 (hD3CDM; Salvatore et al., 1995) were a gift from Dr. P. Reed Larsen (Harvard Medical School, Boston, Massachusetts). Adenovirus plasmids pDeltaE1sp1A, pBHG10, pCA3 and HEK293 cells were purchased from Microbix Biosystems Inc. (Toronto, Canada). DIO3 expression cassette was liberated from parental

plasmids with TaqII (Chimerx, Milwaukee, Wisconsin) and Acc65I (New England Biolabs, Ipswich, Massachuesetts) digests, end filled, gel purified, and blunt end cloned into the EcoRV site of pDeltaE1sp1A. The DIO2 gene was liberated with SacII and Not I, end filled, and blunt end cloned into the EcoRI site of pCA3 which had also been end filled and dephosphorylated. Subclones were isolated and identified by predicted restriction patterns. Adenoviruses expressing deiodinase were constructed by cotransfecting HEK293 cells with the subcloned gene and pBHG10 (Graham, 2000; Graham and Prevec, 1991; Hitt et al., 1994). Isolation of recombinant viruses and subsequent production of DIO2 and DIO3 were identical to DIO1 described earlier (Hornung et al., 2018). Cell lysates were then frozen at −80° C until use in the deiodinase inhibition assays. Protein concentrations were determined using Bradford Reagent with bovine serum albumin as the standard (Sigma-Aldrich). The protein concentration and specific activity varied slightly between the different batches. We previously reported the specific activity of DIO1 to be 9 pmol iodide/h/μg of protein. For DIO2 and DIO3, the specific activities were about 5 and 90 pmol iodide/h/μg of protein, respectively.

Deiodinase Inhibition Assays

Inhibition assay and iodide extraction.—The deiodinase assay methods were first developed for DIO1, closely following Renko et al. (2012, 2015) and are described in Hornung et al. (2018). Briefly, the assay measures deiodinase-liberated iodide with the Sandell-Kolthoff (SK) reaction in a 96-well plate format. The HEK293 cell lysate with expressed DIO1 was thawed, mixed, and diluted in pH 7.0 HEPES buffer. The diluted enzyme (59.4 μl, containing about 10 μg of protein) was then added to the 96-well assay plate (untreated polystyrene, 360 μl well volume, Corning, Corning, New York). Then, 1.2 μl of each test chemical in DMSO was added from the chemical source plate to each well with a Liquidator 96-20 pipettor (Mettler-Toledo Rainin, LLC, Oakland, California) to achieve the final target concentration of 200 μM for each test chemical and 1% DMSO. This maximized the chemical test concentration (given the target concentration on the chemical source plates), and 1% DMSO was found to not inhibit deiodinase enzymes in these assays. The reaction was initiated with the addition of 59.4 μl DIO1 substrate (3,3’,5’-triiodo-L-thyronine, rT3) and dithiothreitol (DTT) in HEPES buffer using a Liquidator 96-200 pipettor (Mettler-Toledo Rainin, LLC). The final assay volume was 120 μl/well with the following conditions: 0.1 M HEPES, 1 mM EDTA, 10 μM rT3, 40 mM DTT, and 1% DMSO. The assay plate was sealed with an adhesive cover sheet (Thermo Fisher Scientific, Waltham, Massachuesetts), mixed in a plate shaker, and incubated for 3 h at 37° C. Following the incubation, 75 μl were transferred to a 96-well, 2 ml polypropylene filtration plate containing Dowex 50WX2 (Biotage USA, Charlotte, North Carolina) and the free iodide was eluted into a 96-well collection plate (Biotage USA) with application of 100 μl of 10% acetic acid.

The DIO2 and DIO3 inhibition assays were adapted from the DIO1 assay described in Hornung et al. (2018) and closely followed methods in Renko et al. (2015), with the following modifications. For DIO2, the HEK293 cell lysate with expressed DIO2 was diluted in pH 7.0 HEPES buffer and then briefly sonicated to obtain a uniform suspension. There were about 20 μg of protein in the 59.4 μl of diluted enzyme added to each well of the

assay plate. The substrate used for DIO2 was T4, and final assay conditions were 0.1 M HEPES, pH 7.0, 1 mM EDTA, 5 μM T4, and 40 mM DTT, 1% DMSO and a final assay volume of 120 μl For DIO3, the HEK293 cell lysate with expressed DIO3 was diluted in pH 8.0 HEPES buffer and sonicated as above to obtain about 1 μg protein in the 59.4 μL of added to each well in the assay plate. The substrate used for DIO3 was T3, and final assay conditions were 0.1 M HEPES, pH 8.0, 1 mM EDTA, 5 μM T3, and 40 mM DTT, 1% DMSO and a final assay volume of 120 μl. Final assay conditions of each assay are included in Supplementary Table 1. The incubation period and temperature, and free iodide elution steps in the DIO2 and DIO3 assays were identical to the DIO1 assay. In the development of the DIO2 and DIO3 assays, two test plates including 18 unique chemicals were used in preliminary concentration-response experiments to verify assay performance. The results from these initial test plates are in Supplementary Tables 2-4.

Sandell-Kolthoff (SK) reaction to detect iodide—In the SK reaction, free iodide catalyzes the reduction of cerium IV (Ce^{+4}) which is yellow-colored to the colorless cerium III (Ce^{+3}) in presence of arsenic (As^{+3}), with the rate of this reaction dependent on the concentration of free iodide (Sandell and Kolthoff, 1937). In all three assays, free iodide was detected using the SK reaction following methods previously described in Hornung et al. (2018). Eluent (75 μl) from the wells of the collection plate was transferred to a new untreated polystyrene 96-well plate. With the Liquidator 96-200 pipet, 75 μl of arsenic reagent [25 mM $NaAsO_2$, 0.8 M NaCl, 0.5 M H_2SO_4] was added and mixed well, followed by the addition of 75 μl of Ce^{+4} reagent [20 mM $(NH_4)_4Ce(SO_4)_42H_20$, 0.44 M H_2SO_4]. Immediately following the addition of the Ce^{+4} reagent, the plate was placed in a Synergy 4 plate reader (BioTek Instruments, Inc., Winooski, Vermont), where it was mixed on the fast setting of the plate reader for three seconds, and then absorbance at 420 nm was read every minute under room temperature. Reaction rate was calculated from the change in absorbance between the 1 and 10 min reading in each well.

Chemical Screening

Screening followed the tiered strategy described in Hornung et al. (2018), with all chemicals tested initially at a single concentration followed by further testing in concentration-response mode for those chemicals that showed greater than 50% inhibition. For DIO1, 26 chemical sources plates were used for single-point screening, with further testing of 142 of these chemicals on 16 concentration-response plates. DIO2 and DIO3 were run in parallel, with 26 plates used in single-point screening and 22 concentration-response plates for further testing of 214 of these chemicals. Before use in the single-point screening, chemical source plates were thawed, and test chemicals were mixed by pipetting action. In addition to the concentration-response curve for the model inhibitors, DMSO and 200 μM of the model inhibitor were each plated into six randomly assigned wells per plate, with the DMSO wells used for maximal deiodinase activity and the model inhibitor wells used for maximal inhibition of activity (see eg, plate map in Supplementary Figure 1). In each of the DIO assays, each chemical source plate was tested on three separate assay plates for n = 3 data points for each chemical.

Chemicals that produced less than 20% inhibition were considered ‘inactive’. Those chemicals that produced 20% inhibition or greater were considered putative deiodinase inhibitors. This 20% threshold was based on the background variability of the maximal activity calculated by three times the DMSO median absolute deviation (DMSO-MAD), which was 12.8, 13.4, and 14.0 across all replicates of all plates for DIO1, DIO2, and DIO3, respectively. Chemicals that produced 50% inhibition or greater were further tested in concentration-response; this level of inhibition had greater separation from the background variability (DMSO control) and is more likely to be biologically-relevant. For concentration-response testing, these chemicals were removed from the original chemical plate and added to new 96-well polypropylene plates (Corning). Dilutions in DMSO were made in this new plate so that they could be tested at final target concentrations of 200, 100, 20, 4.0, 0.8, 0.16, and 0.032 μM (see eg, plate map in Supplementary Figure 2). These concentration-response plates were tested on three separate assay plates for n=3 data points for each concentration of each chemical. Test chemical plates were sealed after use in assays with encapsulated pressure sensitive film (Phenix Research Products, Candler, North Carolina).

Data Processing and Analysis

Data were processed and analyzed using R (version 3.3.1; R Core Team 2016). Data from each plate were processed through an automated pipeline for data normalization, calculation of plate diagnostics, and assay-specific flags. Plate-wise normalization was based on the high concentration of the model inhibitor (200 μM PTU or XTH) and the solvent control (DMSO). Data were processed by the following steps: (1) determine the change in absorbance between the 1- and 10-min readings for each well; (2) calculate the net change in absorbance by then subtracting the mean background change in absorbance defined by the completely inhibited reaction (in six wells containing 200 μM model inhibitor); and (3) normalize to % of control by calculating as a % of the mean net change of the uninhibited reactions (in seven DMSO control wells). Percent inhibition was calculated as 100 minus the percent of the DMSO control reaction. For the single-point screening, median of the three replicates was calculated and results are reported as percent inhibition. The median % inhibition produced by each chemical was then compared across the three deiodinase assays. Chemicals were categorized as producing 50% inhibition or greater in one, two, or three of the deiodinase assays. Hierarchical clustering and heat mapping were used to visualize the similarities and differences in the inhibition produced in this single-point screening, with divisive analysis clustering and plotting with the R packages ‘cluster’ version 2.0.5 (Maechler et al., 2016) and ‘gplots’ version 3.0.1 (Warnes et al., 2016).

Concentration-response data were analyzed with the ToxCast Analysis Pipeline (tcpl) package version 1.0 (Filer et al., 2017) using percent inhibition as the response value and 20% inhibition as the threshold cutoff. This method uses all replicates for each concentration of a chemical to fit dose-response curves based on three models (constant, constrained Hill, and constrained gain-loss model) with the best model selected based on lowest Akaike Information Criterion (AIC) value. For chemicals that fit the robust Hill model, absolute IC_{20}, absolute IC_{50}, and Hill slope were calculated from the model fit parameters provided by the tcpl package. Here we display the concentration-response curves in the negative direction (inhibition from maximum response).

Assay quality/performance—To ensure quality and consistency over more than 100 assay plates run for each DIO assay, quality control measures were calculated for each assay plate. The DMSO control and 200 μM model inhibitor wells were used to evaluate variability of and separation between the positive (model inhibitor) and DMSO (solvent/negative) controls. Both a plate-wise DMSO-MAD and a plate-wise positive control median absolute deviation (200 μM-MAD) were calculated and reported in Table 1. The Z' factor was calculated for each replicate of each assay plate. This metric reflects the dynamic range of the assay and the variability around the maximal and minimal response levels of the assay (Zhang et al., 1999). A Z' factor of 0.5 or greater was used as a guideline for acceptable plate runs. If quality criteria were not met (eg, low Z' factor, poor control data), assay plates were typically rerun to replace the data from the poor plate runs. In several instances, individual replicates of single concentration plates with Z' factor less than 0.5 were not re-run because the other two replicates for those plates met the acceptable quality criteria. For the chemicals that were replicated across multiple plates in single-point screening, the median percent inhibition was compared across all the plates, with the median percent inhibition from testing on chemical source plates for ToxCast ph1v2 used in summaries and analyses.

Test chemical wells or single data points that fell outside of acceptable parameters were automatically flagged for manual review. Flags included high variability or outliers across replicates, no change in absorbance (typically due to a well not receiving one or more reagent), and potential assay interference indicated by absorbance outside of normal ranges. Wells were flagged for high variability when the absolute difference between the mean and median of the three replicate runs was greater than 10%. Potential issues with reagents or assay interference were flagged based on absorbance changing faster than the DMSO control (uninhibited reaction) or slower than the positive control (fully inhibited reaction), with wells flagged when: (a) change in absorbance was less than 0.1 absorbance units; (b) absorbance at one min was less than 85% of the DMSO control; or (c) absorbance at ten min was either less than 50% of the DMSO control or greater than 115% of the positive control. In addition, chemicals were flagged when the median response was greater than 190% or less than −20% of the DMSO control. Wells with observed problems (eg, low volume in a well for one replicate) were excluded from analyses with only two replicates used for these wells as long as variability between the two was less than 20%. Chemicals with high variability across replicates or other anomalous absorbance data were further evaluated with concentration-response testing. Chemicals with evidence of assay interference were excluded from summaries and analyses; such evidence included immediate change to near zero absorbance (typically an indication of the presence of high free iodide), no change in absorbance, and abnormal absorbance time course with values outside the expected range based on the high concentration of the model inhibitor and DMSO control; the 32 chemicals with evidence of assay interference are listed in Supplementary Table 5.

Results

Single-Point, High Concentration Screening

The three deiodinase assays (DIO1, DIO2, and DIO3) were used to assess the ToxCast ph1v2, ph2, and e1k chemical libraries first at a single target concentration of 200 μM (as permissible with solubility). Results and analysis reported here are for 1,819 chemicals due to evidence of assay interference by 32 of the 1,851 chemicals provided (described above in *Assay quality/performance* and listed in Supplementary Table 5). For each chemical, CASRN, the maximum concentration tested, and the median % inhibition produced in single-point screening are included in the Supplementary Table 6. Most of the chemicals tested did not inhibit deiodinase activity (Table 2, Figure 1). In this single concentration initial screen, 1,407 of the chemicals produced less than 20% inhibition in all three DIO assays compared with activity of the DMSO controls. There were 411 chemicals that produced greater than 20% inhibition, of which 228 chemicals produced greater than 50% inhibition in at least one of the DIO assays, which represent 22.5% and 12.5%, respectively, of the chemicals tested.

Concentration-Response Screening

A total of 240 chemicals were tested in concentration-response mode, with 142 chemicals tested in the DIO1 assay and 213 chemicals tested in the DIO2 and DIO3 assays. This concentration-response testing included the chemicals that produced 50% or greater inhibition in the single-point screening in each assay (see summary in Table 2) as well as a subset of those chemicals that produced less than 50% inhibition. These subsets were included as an additional quality control for intra-assay consistency and consisted of 7, 55, and 38 chemicals, respectively, for DIO1, DIO2, and DIO3. For the DIO1 assay, this subset included chemicals with high variability or other issues that were encountered in single-point screening. Because the DIO2 and DIO3 assays were run in parallel, these subsets included the chemicals that produced inhibition of 50% or greater in only one of these two assays. Supplementary Table 7 includes all chemicals that were tested in concentration-response, with sources, chemical name, CASRN, the maximum concentration tested, median % inhibition produced at the maximum tested concentration, and Hill model fit parameters, where appropriate.

The concentration-response results confirmed the single-point results for over 97% of these chemicals. Results were considered consistent when there was less than 25% difference between the median inhibition produced in the single-point screening and that produced at the maximum concentration tested in concentration-response, and the chemical was categorized as ‘inactive’ or a putative deiodinase inhibitor in both tests. In the DIO1 assay, the concentration-response results were consistent with single-point results for 139 of the 142 chemicals tested and only one chemical (chlorophene) with a difference in categorization. Chlorophene had high variability across the multiple single-point plates on which it was tested, producing a range of inhibition in from 11% to 47% (Supplementary Figure 3), but produced less than 20% inhibition in concentration-response testing. In the DIO2 assay, 209 of the 213 chemicals had consistent single-point and concentration-response results, with three chemicals (1-dodecanol, 1-tridecanol, and 1-dodecanamine) that

produced 35% inhibition or greater in single-point screening and less than 7% inhibition in concentration-response testing. In the DIO3 assay, concentration-response results for 207 of the 213 chemicals were consistent with the single-point screening results. The six chemicals with differences in the DIO3 assay all consistently produced inhibition greater than 20% and the single-point and concentration-response results differed by 26%-40% (eg, 29% vs 55% inhibition or 67% vs 97% inhibition). Several chemicals were identified as potential false negatives in the DIO2 and DIO3 assays, where the chemical produced less than 20% inhibition in the single-point screening but 20% or greater in the concentration-response testing. However, the maximum % inhibition produced by these chemicals was just above the 20% cut-off and, therefore, these chemicals were considered only borderline active.

The inhibition curves for all chemicals tested in concentration-response mode are available in Supplementary Figure 5 with percent inhibition at maximum tested concentration and Hill model results (Hill slope, absolute IC_{20}, and absolute IC_{50}, when applicable) included in Supplementary Table 7. Hill model parameters are reported for all chemicals that produced inhibition of 20% or greater at the maximum concentration tested in concentration-response; this model was identified in the ToxCast pipeline as the best fit model for most of these chemicals (135 of the 136 chemicals in DIO1, all 192 chemicals in DIO2, and 196 of the 199 chemicals in DIO3) and an acceptable model fit for the rest of these chemicals

For each assay, the top 25 ranked chemicals, based on absolute IC_{50}, are included in Tables 3–5. In the DIO1 assay, nine chemicals had IC_{50} lower or comparable with the mean IC_{50} for the control PTU (5.4 μM), including the ToxCast plated PTU and the known DIO1 inhibitor genistein. In the DIO2 assay, no chemical produced an IC_{50} near that of the mean IC_{50} for the control chemical XTH (0.8 μM), with the closest chemicals at 2.2 μM (fluazinam), 2.9 μM (triflumizole), and 3.7 μM (bisphenol A diglycidyl ether). In the DIO3 assay, there were two chemicals (nordihyroguaiaretic acid and chlorothalonil) with an IC_{50} lower or comparable with that of the control XTH (0.3 μM).

Comparison across deiodinases

The inhibition results were similar across the three deiodinases for over 90% of the tested chemicals. In single-point screening, 1,590 chemicals produced less than 50% inhibition in all three assays (Supplementary Table 6). Of the 228 chemicals that produced inhibition of 50% or greater in one or more of the assays, 93 chemicals produced this result in all three assays (Figure 2). This comparison also identified 54 chemicals that inhibited only two of the three deiodinases (DIO1/DIO2 = 11, DIO2/DIO3 = 34, and DIO1/DIO3 = 9 chemicals) and isoform-specific inhibition by 81 chemicals that produced inhibition of 50% or greater in only one of the three deiodinase assays (DIO1=22, DIO2=20, and DIO3=39 chemicals). Concentration-response testing also showed similarities and differences in chemical inhibition of the three deiodinases, with inhibition curve shapes nearly identical for some and differing greatly for other chemicals, as shown with five example chemicals in Figure 3.

Assay performance and quality control

Performance of the assay was monitored in each assay plate through Z' factor, variability of the positive and solvent controls, and IC_{50} for the model inhibitor (Table 1). All three assays

performed reproducibly with excellent dynamic range, low variability of the control chemicals, and consistent responses of the model inhibitor concentration-response curves on each plate. The Z' factor was generally near 0.7 for individual replicates of each assay plate and mean Z' factor was above 0.5 for all single-point and concentration-response plates, with the exception of one single-point DIO2 plate (mean Z' factor = 0.4). The plate-wise MADs for the DMSO control and the 200 μM model inhibitor wells were 10% or less for nearly all of assay plates (93% of DIO1 and 95% of DIO2 and DIO3 assay plates). The concentration-response inhibition curves for the model inhibitors were consistent across plates within each assay (see eg, in Supplementary Figure 4). The IC_{50} values ranges were 3.1–8.9 μM PTU for DIO1, 0.4–1.1 μM XTH for DIO2, and 0.1–0.9 μM XTH for DIO3.

The ten chemicals used for intra-assay reproducibility had very consistent results across multiple plates (Supplementary Figure 3). In each assay, there were four to six chemicals that consistently produced less than 20% inhibition and two to five chemicals that produced inhibition of 20% or greater. These chemicals had highly reproducible results with the exceptions of chlorophene and PFOS in DIO1, which could possibly be explained by chemical degradation or different lots of a chemical provided on the chemical source plates.

Chemicals with potential assay interference

The 32 chemicals that were flagged as potentially interfering in these DIO assays are listed in the Supplementary Table 5. The assay results for these chemicals fell outside of acceptable parameters as described above (in the Materials and Methods section). Several of these chemicals are from chemical classes with known interferences with the SK reaction (eg, thiocyanate, Sandell and Kolthoff, 1937). Iodine-containing chemicals may act as substrates for deiodinases (eg, iopanoic acid, Renko et al., 2012) or may have free iodide that causes false results in these assay (eg, sodium iodide). These chemicals were excluded from summaries and analyses; further evaluation of the source of interference in each of these chemicals was outside of the scope of this study.

Discussion

The extensive screening effort presented here identified 411 putative deiodinase inhibitors through testing 1,819 chemicals at a single, high concentration in recently developed 96-well plate assays described in Hornung et al. (2018) for DIO1 and here for DIO2 and DIO3. Of these, 228 chemicals produced inhibition of 50% or greater in one or more of the deiodinases including chemicals that, to our knowledge, have not previously been known to inhibit deiodinases. In addition, the single-point and concentration-response results suggest differential inhibition with varied maximum inhibition and potency for some chemicals when compared across DIO1, DIO2, and DIO3.

The consistent performance in these assays demonstrated their reproducibility and fitness for screening and prioritizing chemicals for potential thyroid disruption. Across multiple months of screening, each assay maintained low variability in controls, reproducible inhibition curves for model inhibitors, high Z' factors, and reliable results across replicated chemicals. Although there are currently no established reference chemicals for deiodinase inhibition, the results from these assays are consistent with earlier studies. PTU, methylthiouracil, and

genistein produced DIO1-specific inhibition, aurothioglucose and xanthohumol inhibited all three DIO isoforms (data not shown for DIO1), and previously confirmed non-inhibitors such as methimazole and bisphenol A were also identified as inactive in these assays (Renko et al., 2015; Tuarog et al., 1994; Visser et al., 1992; Wassen, et al., 2004). In addition, these assay results match studies that identified some halogenated phenolic compounds (Butt et al., 2011) and flavonoids (Auf'mkolk et al., 1986; Ferreira et al., 2002; Spanka et al., 1990) as inhibitors of DIO1 enzyme activity.

Results from *in vitro* screening assays have limitations and uncertainties inherent to them, including nonspecific chemical activity and assay interference (Judson et al., 2013, 2016; Thorne et al., 2010). Chemicals producing disruption by these means could be identified through a comparison across multiple *in vitro* assays. Of the 93 chemicals with greater than 50% inhibition in all three deiodinases assays, over 20 chemicals were identified as surfactants (eg, sodium dodecyl sulfate, hexadecyltrimethyl-ammonium bromide) or related chemicals (eg, linoleic acid), which may disrupt membranes, the test system, or be related to nonspecific enzyme inhibition. However, this set of chemicals that produced greater than 50% inhibition in all three assays included a range of chemical types and further analysis is required to differentiate false positives from specific deiodinase inhibition. The screening strategy of initial testing with a single concentration has a potential risk of false negatives, as active compounds can only be defined based on highest concentration tested. Ideally, the tested concentration would be consistent across all chemicals; however chemical solubility and purity, and protein sequestration of chemical could alter actual tested concentrations. In these deiodinase assays, 96% of the chemicals were tested in single-point at or near the target concentration of 200 μM (150 – 210 μM) and efforts were taken to reduce the possibility of chemical degradation (eg, limiting number of freeze/thaw cycles); however, 19 chemicals were initially tested at less than 100 μM (see Supplementary Table 6 for tested concentrations of all chemicals). We also employed a set of interference flags specific to these deiodinase assays to identify chemicals that were producing false positives/negatives. With this data-based approach, we identified 32 compounds with evidence of interfering with one or more of the assays, as described in the Results section above and listed in Supplementary Table 5. This set of interfering compounds included both potential false negatives and false positives, all of which were excluded from assay results and summaries. However, further testing with other methods (eg, monitoring for changes in substrate with liquid chromatography-tandem mass spectrometry) is warranted for these chemicals, as several are suspected thyroid disruptors or have been previously documented to produce inhibition in deiodinases (eg, Tetrac, FD&C Red 3, amiodarone hydrochloride, iopanoic acid) (Bartalena et al., 2018; Braga and Cooper, 2001; Capen and Martin, 1989; Koehrle et al., 1986; Renko et al. 2012, 2015; Rosene et al., 2010).

These data provide the most comprehensive examination of chemical inhibition of deiodinase activity currently available, greatly expanding on previous studies that included ten to fifteen compounds for one or more deiodinases (eg, Butt et al., 2011; Ferreira et al., 2002; Renko et al., 2015), as well as our recent report on approximately 300 chemicals for inhibition of DIO1 (Hornung et al. 2018). The chemicals that produced maximum inhibition and potency in each assay are of most interest. In the top 25 chemicals in each assay, we identified 12 chemicals for DIO1 and 2 chemicals each for DIO2 and DIO3 that exhibited

potency similar or greater than the model inhibitors based on IC_{50}. Few of these most potent chemicals, however, have been reported in the literature to inhibit deiodinases. The few chemicals previously reported to produce deiodinase inhibition were only on the list of potent chemicals in the DIO1 assay. These included the well-documented inhibition of DIO1 produced by genistein, PTU, and 6-methyl-2-thiouracil (Kaplan and Utiger, 1978; Renko et al., 2015; Schweizer and Steegborn, 2015; Visser et al., 1992; Wassen et al., 2004). In addition, Chopra et al. (1985) reported inhibition of the conversion of T4 to T3 by fatty acids, including linoleic acid, and Ferreira et al. (2002) demonstrated that morin reduced thyroid DIO1 activity. Previous reports of deiodinase inhibition are limited for these most potent chemicals; however, several of the potent deiodinase inhibitors in this study have been documented to affect thyroid axis-endpoints. Two examples are fipronil, which is suspected to enhance hepatic metabolism and excretion of TH (Hurley et al., 1998; Leghait et al., 2009), and nordihydroguaiaretic acid, which has been shown to inhibit growth of thyroid cells (Gartner et al. 1985). While a comprehensive review of the literature for potential thyroid disruption by the chemicals producing deiodinase inhibition in these assays is warranted, it is beyond the scope of this study. In addition, targeted *in vivo* testing is needed to expand the limited understanding of how *in vitro* inhibition assay results relate to effects of inhibition of deiodinase activity *in vivo*.

Results from this set of three assays provide a unique opportunity to compare chemical inhibition across the deiodinase isoforms for many chemicals. In the set of 93 chemicals that produced inhibition of 50% or greater in all three assays, we recognized the potential for nonspecific enzyme inhibition. However, activity across multiple deiodinase assays could also be used as a method to prioritize chemicals for further investigation as chemicals disrupting multiple molecular targets on the thyroid-axis could result in more extreme adverse organismal outcomes. This will be especially informative when it is possible to conduct a comprehensive comparison across a suite of thyroid-relevant *in vitro* assays. This could include the deiodinase results reported here, the recently completed screening for NIS inhibition (Wang et al., 2018), and the TPO, TH receptor transactivation, and thyrotropin releasing hormone results previously completed for these chemical libraries (Martin et al., 2010; Paul Friedman et al., 2016; Sipes et al., 2013). Chemicals with differences across the deiodinases are also of great interest, especially the 81 chemicals that produced inhibition of 50% or greater in only one of the three deiodinases. This set of assays identified 22 chemicals that produced inhibition of 50% or greater in DIO1 but not in DIO2 or DIO3, including the known DIO1-specific inhibitors PTU, 6-methyl-2-thiouracil, and genistein (Renko et al., 2015; Schweizer and Steegborn, 2015; Wassen et al., 2004). In addition, 20 chemicals were identified as possible DIO2-specific inhibitors, and 39 chemicals were identified as possible DIO3-specific inhibitors. An initial examination of classes of the chemicals with differential inhibition showed potentially interesting patterns. For example, 11 of the 23 chemicals classified as triazines (aromatic or aliphatic) in ECOSAR version 2.0 (U.S. EPA, 2017b) produced inhibition greater than 20% in DIO3 but not DIO1 or DIO2, and four of these chemicals produced DIO3-specific inhibition greater than 50%. As another example, the three closely related tamoxifen compounds (tamoxifen, tamoxifen citrate, and 4-hydroxytamoxifen) each produced almost complete inhibition in DIO1 and DIO2, but not DIO3 (Figure 3). To our knowledge, these chemical class-specific differences in deiodinase

inhibition have not previously been reported and provide the impetus for the development of structure-activity relationship models.

In summary, this set of three deiodinase inhibition assays is a significant contribution towards expanding the limited number of *in vitro* assays used to identify chemicals having the potential to interfere with TH homeostasis. Of the 1,819 chemicals tested, 22% were identified as putative deiodinase inhibitors, with between 12% and 17% in each assay. This study sets the groundwork for development and evaluation of structure-activity relationships for deiodinase inhibition, and informs targeted selection of chemicals for further testing to evaluate effects on THs and identify adverse outcomes of deiodinase inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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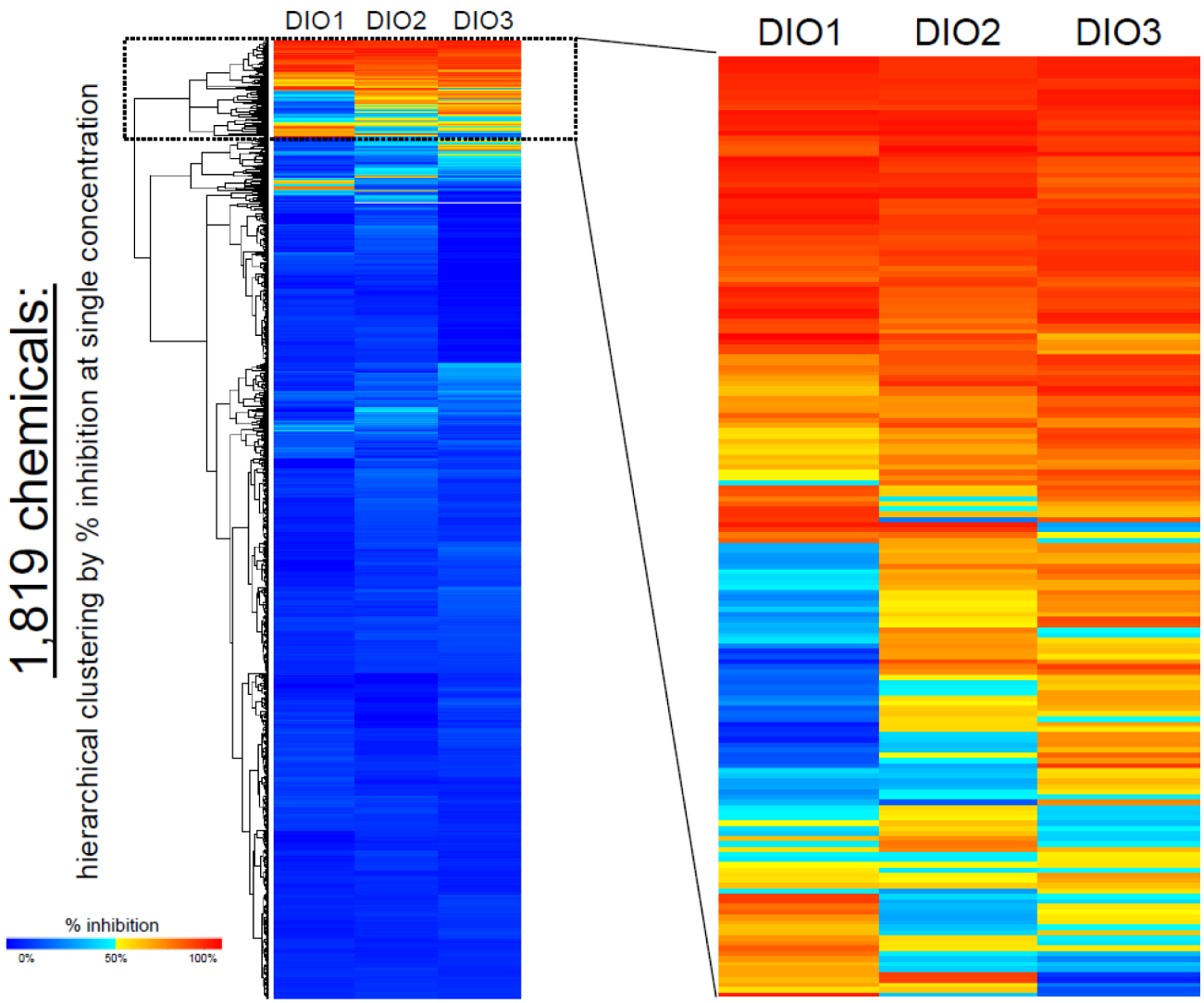

Figure 1.

Deiodinase inhibition produced by the 1,819 chemicals tested in single-point screening (target concentration of 200 μM), ordered by hierarchical clustering (divisive analysis method), with expanded view of 180 chemicals. Median of n = 3 replicates. The tested concentration and median % inhibition values for each chemical are in Supplementary Table 6.

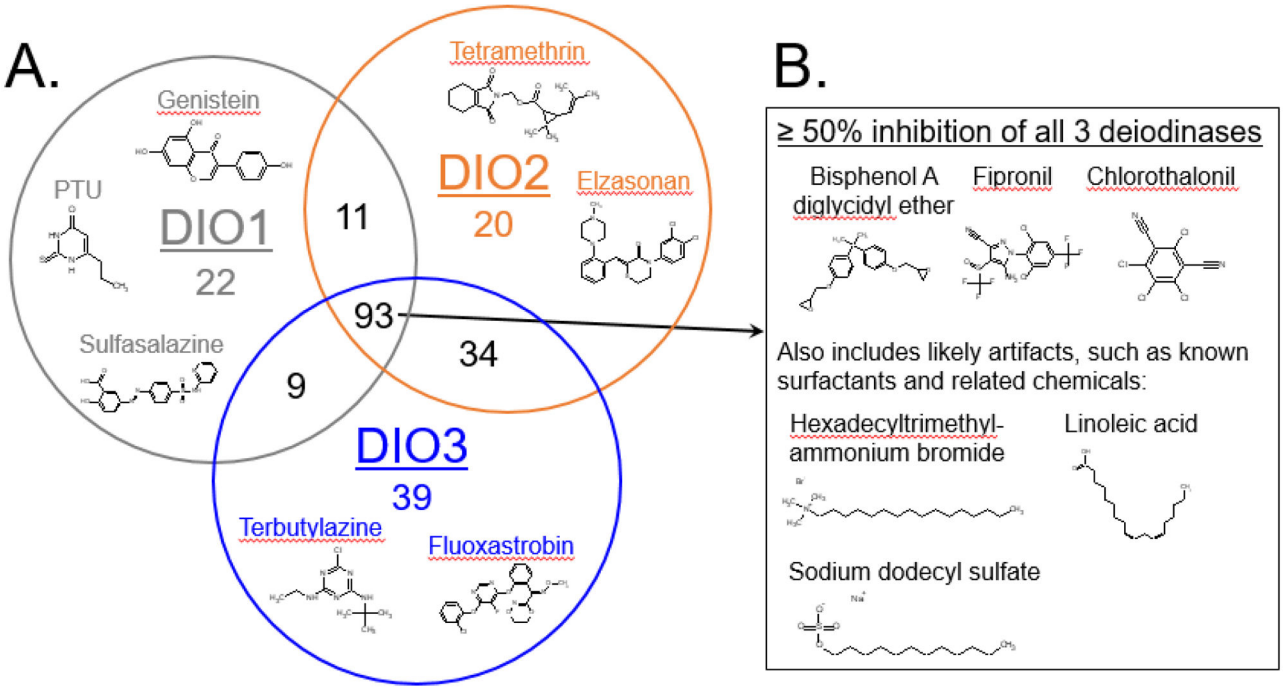

Figure 2.

A, The number of chemicals that produced 50% inhibition or greater in one or more deiodinase assay (Venn diagram) with examples that produced ≥ 50% inhibition specific to a single enzyme; and B, examples that produced ≥ 50% inhibition in all three deiodinases.

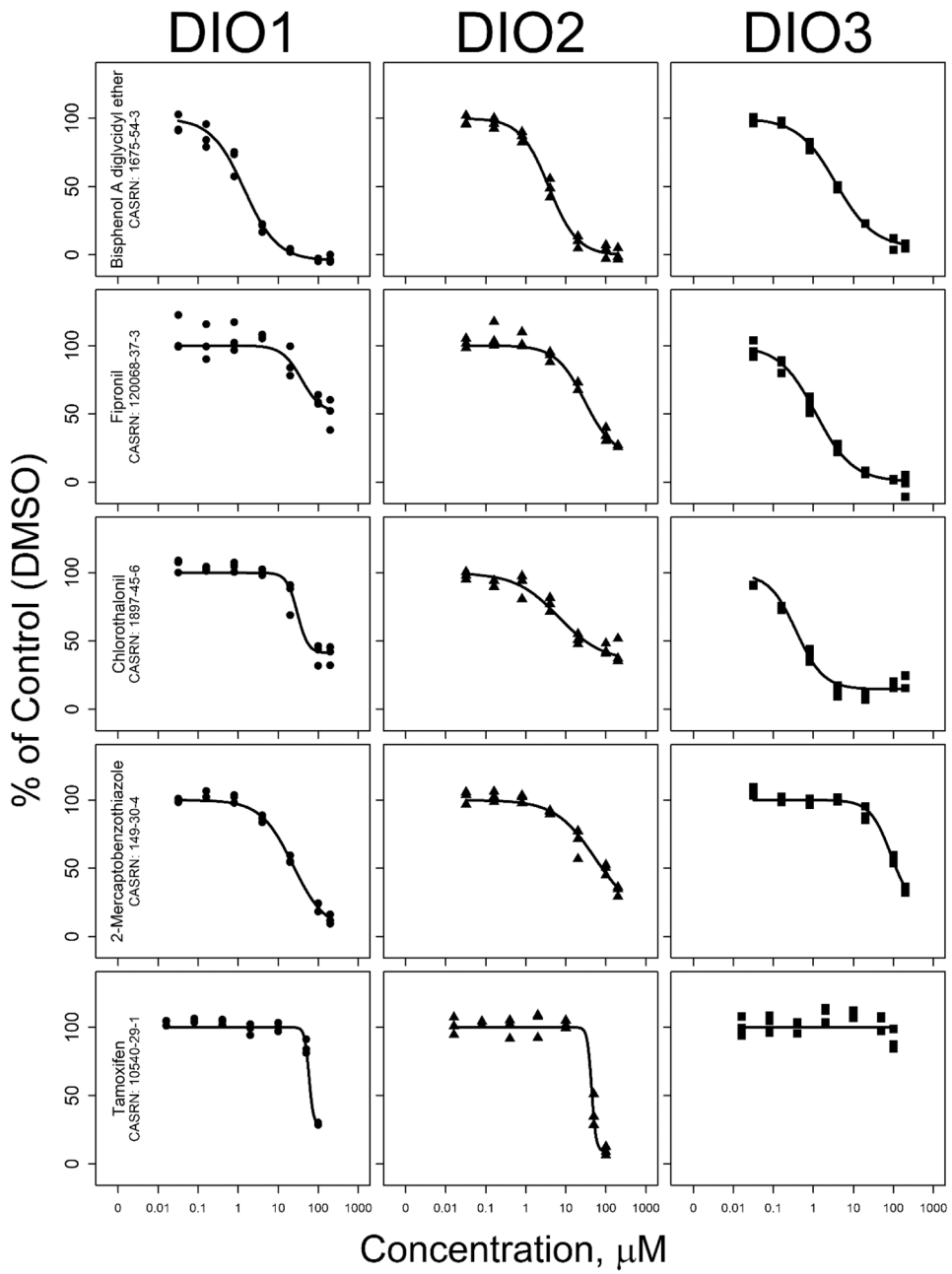

Figure 3.

Concentration-response curves for deiodinase inhibition by five example compounds, tested at seven concentrations.

Table 1.

Summary of the concentration-response curves for the model inhibitors and assay performance across the test chemical plates for both single-point (single high concentration) and concentration-response testing.

	Model Inhibitor						DMSO	Z' factor[d]
Enzyme	**Chemical Name**	**CASRN**	**Inhibition Curve Concentrations, µM**	**IC_{50}[a], µM Mean ± SD**	**Hill slope[a] Mean ± SD**	**200 µM-MAD[b] Median**	**MAD[c] Median**	**Mean ± SD**
DIO1	6-Propyl-2-thiouracil	51-52-5	0.033 – 1000 [e]	5.4±1.3	−1.3±0.5	3.15	4.12	0.7±0.1
DIO2	Xanthohumol	6754-58-1	0.0002 – 200 [f]	0.8±0.2	−0.9±0.3	3.06	3.85	0.7±0.1
DIO3	Xanthohumol	6754-58-1	0.0002 – 200 [f]	0.3±0.1	−0.9±0.1	2.48	4.18	0.7±0.1

[a] IC_{50} and Hill slope from four-parameter logistic model was calculated from the three replicate runs of each assay plate. Mean and SD across assay plates are reported above for each DIO. Each assay had 26 single-point plates with 16 concentration-response plates for DIO1 and 22 concentration-response plates for DIO2 and DIO3; IC_{50} and Hillslope not calculated for one DIO2 plate due to errors in the diluted XTH wells.

[b] Model inhibitor-MAD is the platewise positive control median absolute deviation calculated from all the 200 µM wells of the model inhibitor in each of the replicated assay plates.

[c] DMSO-MAD is the platewise median absolution deviation calculated from all the DMSO wells in each of the replicated assay plates.

[d] Z' factor is a measure of assay quality, with value of >0.5 indicating good separation between the positive and negative controls.

[e] PTU inhibition curve concentrations: 0.033, 0.33, 1, 3.3, 10, 33, and 1000 µM in column A, with 6 wells of 200 µM distributed across each plate.

[f] XTH inhibition curve concentrations: 0.0002, 0.002, 0.02, 0.2, 2, 20, and 200 µM in column A, with 6 additional wells of 200 µM distributed across each plate.

Table 2.

Summary of the single-point screening at an initial target concentration of 200 μM: percent and number of chemicals in each library that produced 20% and 50% inhibition.

Chemical Library	No. Chemicals Tested[a]	Deiodinase Type 1 (DIO1)		Deiodinase Type 2 (DIO2)		Deiodinase Type 3 (DIO3)	
		% With ≥ 20% Inhibition (#)	% With ≥ 50% Inhibition (#)	% With ≥ 20% Inhibition (#)	% With ≥ 50% Inhibition (#)	% With ≥ 20% Inhibition (#)	% With ≥ 50% Inhibition (#)
ToxCast ph1_v2	290	16.9 % (49)	6.5 % (19)	18.6 % (54)	8.6 % (25)	19.7 % (57)	9.7 % (28)
ToxCast ph2	749[b]	12.7 % (95)	8.0 % (60)	16.8 % (126)	9.0 % (67)	15.6 % (117)	9.2 % (69)
ToxCast e1k	780	9.9 % (77)	7.2 % (56)	15.8 % (123)	8.5 % (66)	17.1 % (133)	10.0 % (78)
Total	1,819[b]	**12.1 % (221)**	**7.4 % (135)**	**16.7 % (303)**	**8.7 % (158)**	**16.9 % (307)**	**9.6 %** (175)

The chemicals names, CAS Registry Number (CASRN), maximum tested concentrations, and median % inhibition produced in each of the three deiodinase assays can be found in Supplementary Table 6.

[a]Chemical plates received from ToxCast included 1,851 unique chemicals, however 32 compounds had evidence of interfering with one or more of the deiodinase assays and thus were excluded from all summaries and analyses.

[b]One chemical from ToxCast ph2 was only tested in DIO1 because it was depleted from the chemical inventory, thus a total of 1,818 chemicals were tested in the DIO2 and DIO3 assays.

Table 3.

The top 25 ranked chemicals for inhibition of deiodinase type 1 (DIO1), based on absolute IC_{50}, with activity across the deiodinases (DIOs) indicating which chemicals produced 50% inhibition or greater in each of the deiodinase assays in single-point screening.

			DIO1				Across DIOs
Rank	Chemical	CASRN	Max Tested Conc., μM	% Inhibition at Max Conc.[a]	IC_{20} (μM)	IC_{50} (μM)	≥ 50% Inhibition
1	4,5-Dichloro-3H-1,2-dithiol-3-one	1192-52-5	200	100	0.1	0.2	DIO 1, 2, 3
2	4,5-Dichloro-2-octyl-3(2H)-isothiazolone	64359-81-5	200	99	0.2	0.4	DIO 1, 2, 3
3	C.I. Direct Yellow 12	2870-32-8	200	103	0.2	0.8	DIO 1, 2, 3
4	Bisphenol A diglycidyl ether	1675-54-3	200	104	0.4	1.3	DIO 1, 2, 3
5	Temephos	3383-96-8	200	93	0.1	1.3	DIO 1
6	Genistein[b]	446-72-0	200	95	0.8	2.6	DIO 1
7	6-Hydroxy-2-naphthyl disulfide	6088-51-3	200	93	0.5	2.7	DIO 1, 2, 3
8	6-Propyl-2-thiouracil[b]	51-52-5	200	101	1.1	3.8	DIO 1
9	C12-14-Alkyl glycidyl ether	68609-97-2	119	65	0.8	4.1	DIO 1, 2
10	Darbufelone mesylate	139340-56-0	100	74	1.4	4.7	DIO 1, 2, 3
11	Morin hydrate	654055-01-3	200	94	0.6	7.4	DIO 1, 3
12	Linoleic acid	60-33-3	200	96	0.7	10.4	DIO 1, 2, 3
13	Nordihydroguaiaretic acid	500-38-9	200	80	1.6	11.0	DIO 1, 2, 3
14	Tributyltetradecylphosphonium chloride	81741-28-8	200	94	5.5	12.4	DIO 1, 2, 3
15	Captafol	2425-06-1	200	94	4.8	12.9	DIO 1, 2, 3
16	Hexadecyltrimethylammonium bromide	57-09-0	200	96	5.9	12.9	DIO 1, 2, 3
17	Chlorhexidine diacetate	56-95-1	200	87	1.2	13.0	DIO 1, 2, 3
18	Zoxamide	156052-68-5	200	98	8.2	17.0	DIO 1, 2, 3
19	Didecyldimethylammonium chloride	7173-51-5	200	106	4.9	17.1	DIO 1, 2, 3
20	Tannic acid	1401-55-4	60	96	9.7	17.4	DIO 1, 2, 3
21	6-Methyl-2-thiouracil[b]	56-04-2	200	88	3.9	18.4	DIO 1
22	Triflumizole	68694-11-1	200	74	13.4	18.8	DIO 1, 2, 3
23	2-Chloro-N-phenylacetamide	587-65-5	200	105	7.0	18.8	DIO 1
24	2-Ethylhexyl acrylate	103-11-7	200	88	3.9	19.4	DIO 1, 2, 3

			DIO1				Across DIOs
Rank	Chemical	CASRN	Max Tested Conc., μM	% Inhibition at Max Conc. [a]	IC_{20} (μM)	IC_{50} (μM)	≥ 50% Inhibition
25	Linolenic acid	463-40-1	200	94	5.5	20.0	DIO 1, 2, 3

[a] Median of three replicates at maximum concentration of chemical in concentration-response testing; inhibition in single-point results differed by 15% or less.

[b] Previously reported to inhibit DIO1 (Renko et al., 2015; Visser et al., 1992; Wassen et al., 2004).

Table 4.

The top 25 ranked chemicals for inhibition of deiodinase type 2 (DIO2), based on absolute IC_{50}, with activity across the deiodinases (DIOs) indicating which chemicals produced 50% inhibition or greater in each of the deiodinase assays in single-point testing.

			DIO2				Across DIOs
Rank	**Chemical**	**CASRN**	**Max Tested Conc., μM**	**% Inhibition at Max Conc.[a]**	**IC_{20} (μM)**	**IC_{50} (μM)**	**≥ 50% Inhibition**
1	Fluazinam	79622-59-6	180	100	0.5	2.2	DIO 1, 2, 3
2	Triflumizole	68694-11-1	200	98	0.7	2.9	DIO 1, 2, 3
3	Bisphenol A diglycidyl ether	1675-54-3	200	101	1.2	3.7	DIO 1, 2, 3
4	Octhilinone	26530-20-1	200	79	3.4	12.2	DIO 1, 2, 3
5	Zoxamide	156052-68-5	200	95	3.1	12.9	DIO 1, 2, 3
6	Chlorhexidine diacetate	56-95-1	200	99	5.7	13.1	DIO 1, 2, 3
7	SSR69071	344930-95-6	50	77	4.5	13.8	DIO 2, 3
8	4,5-Dichloro-3H-1,2-dithiol-3-one	1192-52-5	200	92	3.0	15.6	DIO 1, 2, 3
9	4,5-Dichloro-2-octyl-3(2H)-isothiazolone	64359-81-5	200	95	2.4	16.9	DIO 1, 2, 3
10	Tributyltetradecylphosphonium chloride	81741-28-8	200	97	9.0	17.8	DIO 1, 2, 3
11	Dinocap	39300-45-3	200	93	3.2	18.2	DIO 1, 2, 3
12	(Dicyclopentadienyloxy)ethyl methacrylate	68169-03-9	200	83	4.4	18.4	DIO 2, 3
13	Isooctyl acrylate	29590-42-9	200	96	4.6	18.7	DIO 1, 2, 3
14	Quinoxyfen	124495-18-7	200	63	4.9	18.7	DIO 2, 3
15	Hydramethylnon	67485-29-4	200	86	16.3	19.8	DIO 2
16	Hexadecyltrimethylammonium bromide	57-09-0	200	101	16.6	19.9	DIO 1, 2, 3
17	2-Ethylhexyl acrylate	103-11-7	200	88	3.0	20.1	DIO 1, 2, 3
18	Methyltrioctylammonium chloride	5137-55-3	200	99	12.4	20.8	DIO 1, 2, 3
19	6-Hydroxy-2-naphthyl disulfide	6088-51-3	200	85	2.7	21.1	DIO 1, 2, 3
20	Benzalkonium chloride	8001-54-5	200	100	16.7	22.5	DIO 1, 2, 3
21	Dichlone	117-80-6	200	94	4.0	22.6	DIO 1, 2, 3
22	AVE5638	725228-45-5	200	96	5.0	22.8	DIO 1, 2, 3
23	Hexachlorocyclopentadiene	77-47-4	200	87	6.0	25.5	DIO 1, 2, 3
24	Calcium dodecylbenzene sulfonate	26264-06-2	200	94	16.5	25.6	DIO 1, 2, 3
25	Ascorbyl palmitate	137-66-6	200	97	6.9	26.4	DIO 1, 2, 3

[a]Median of three replicates at maximum concentration of chemical in concentration-response testing; inhibition in single-point results differed by 12% or less, except for dinocap, which produced 49.4% inhibition.

Table 5.

The top 25 ranked chemicals for inhibition of deiodinase type 3 (DIO3), based on absolute IC_{50}, with activity across the deiodinases (DIOs) indicating which chemicals produced 50% inhibition or greater in each of the deiodinase assays in single-point screening.

			DIO3				Across DIOs
Rank	Chemical	CASRN	Max Tested Conc., µM	% Inhibition at Max Conc.[a]	IC_{20} (µM)	IC_{50} (µM)	≥ 50% Inhibition
1	Nordihydroguaiaretic acid	500-38-9	200	97	0.01	0.1	DIO 1, 2, 3
2	Chlorothalonil	1897-45-6	200	76	0.1	0.5	DIO 1, 2, 3
3	Pentachloropyridine	2176-62-7	200	98	0.2	1.0	DIO 2, 3
4	Fipronil	120068-37-3	200	101	0.3	1.2	DIO 1, 2, 3
5	Quinoxyfen	124495-18-7	200	88	0.2	1.3	DIO 2, 3
6	Hexadecyltrimethylammonium bromide	57-09-0	200	97	1.0	1.6	DIO 1, 2, 3
7	AVE6324	NOCAS_47377	200	86	0.4	1.8	DIO 1, 2, 3
8	Fluazinam	79622-59-6	180	101	0.4	2.0	DIO 1, 2, 3
9	Triflumizole	68694-11-1	200	102	0.5	2.3	DIO 1, 2, 3
10	Ergocalciferol	50-14-6	200	95	0.5	2.6	DIO 1, 2, 3
11	Chlorhexidine diacetate	56-95-1	200	103	1.3	2.6	DIO 1, 2, 3
12	6-Hydroxy-2-naphthyl disulfide	6088-51-3	200	102	0.6	3.1	DIO 1, 2, 3
13	Octadecyl sulfate sodium salt	1120-04-3	200	93	1.7	3.3	DIO 1, 2, 3
14	Hexachlorocyclopentadiene	77-47-4	200	99	0.7	3.3	DIO 1, 2, 3
15	Didecyldimethylammonium chloride	7173-51-5	200	100	2.8	3.3	DIO 1, 2, 3
16	4,5-Dichloro-3H-1,2-dithiol-3-one	1192-52-5	200	103	0.7	3.3	DIO 1, 2, 3
17	Tributyltetradecylphosphonium chloride	81741-28-8	200	81	2.8	3.3	DIO 1, 2, 3
18	1,2-Benzenedicarboxaldehyde	643-79-8	200	93	0.7	3.5	DIO 1, 2, 3
19	Methyltrioctylammonium chloride	5137-55-3	200	89	3.2	3.8	DIO 1, 2, 3
20	2-(8-Heptadecenyl)-2-imidazoline-1-ethanol	95-38-5	200	101	3.3	3.9	DIO 1, 2, 3
21	Bisphenol A diglycidyl ether	1675-54-3	200	94	0.9	4.1	DIO 1, 2, 3
22	Ascorbyl palmitate	137-66-6	200	98	1.2	4.2	DIO 1, 2, 3
23	Kepone	143-50-0	200	100	2.2	4.4	DIO 1, 2, 3
24	2-Benzylideneoctanal	101-86-0	200	84	1.1	4.7	DIO 2, 3
25	SSR69071	344930-95-6	50	90	1.6	5.1	DIO 2, 3

[a]Median of three replicates at maximum concentration of chemical in concentration-response testing; inhibition in single-point results differed by 11% or less except quinoxyfen, which produced 49.4% inhibition, and 2-benzylideneoctanal which produced 65.0% inhibition.