Screening the ToxCast Chemical Libraries for Binding to Transthyretin

Stephanie A. Eytcheson[1,2], Alexander D. Zosel[2,3], Jennifer H. Olker[2], Michael W. Hornung[2], Sigmund J. Degitz[2*]

[1]Oak Ridge Institute for Science and Education Postdoctoral Fellow, Oak Ridge, TN, USA
[2]U.S. Environmental Protection Agency, Office of Research and Development, Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division, Duluth, MN, USA
[3]Oak Ridge Associated Universities Student Services Contractor, Oak Ridge, TN, USA

Disclaimer: *The views expressed in this paper are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.*

[*]To whom correspondence should be addressed at U.S. EPA, Office of Research and Development, Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division, 6201 Congdon Blvd, Duluth, MN 55804 USA. E-mail: degitz.sigmund@epa.gov Tel: 218-529-5168

Conflict of Interest: The authors claim no conflicts of interest.

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

Abstract

Transthyretin (TTR) is one of the serum binding proteins responsible for transport of thyroid hormones (TH) to target tissue and for maintaining the balance of available TH. Chemical binding to TTR and subsequent displacement of TH has been identified as an endpoint in screening chemicals for potential disruption of the thyroid system. To address the lack of data regarding chemicals binding to TTR, we optimized an *in vitro* assay utilizing the fluorescent probe 8-anilino-1-napthalenesulfonic acid (ANSA) and the human protein TTR to screen over 1,500 chemicals from the U.S. EPA's ToxCast ph1_v2, ph2, and e1k libraries utilizing a tiered approach. Testing of a single high concentration (target 100 µM) resulted in 888 chemicals with 20% or greater activity based on displacement of ANSA from TTR. Of these, 282 chemicals had activity of 85% or greater and were further tested in 12-point concentration-response with target concentrations ranging from 0.015-100µM. An EC50 was obtained for 276 of these 301 chemicals. To date, this is the largest set of chemicals screened for binding to TTR. Utilization of this assay is a significant contribution towards expanding the suite of *in vitro* assays used to identify chemicals with the potential to disrupt thyroid hormone homeostasis.

Keywords: thyroid, screening, endocrine disruption, transthyretin, NAMs, *in vitro*

Abbreviations: TTR, transthyretin; NAMs, new approach methodologies; EDCs, endocrine disrupting compounds; TH, thyroid hormone

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

23 **Introduction**

24 Thousands of chemicals are developed each year adding to the thousands of chemicals
25 already in use (Naidu et al., 2021; van Dijk et al., 2021). It has been demonstrated that exposure
26 to environmental contaminants can have adverse biological impacts; thus, it is necessary to
27 characterize the risks of these chemicals (Lazofsky & Buckley, 2022; Tijani et al., 2016). As the
28 number of chemicals grows each year, it becomes increasingly prohibitive to test them for
29 effects *in vivo*. As such, regulatory agencies are turning to new approach methodologies
30 (NAMs), including *in vitro* high throughput screening, to assess a chemical's potential to interact
31 at a specific molecular target and to identify endocrine disrupting chemicals (EDCs) (Mondou et
32 al., 2020; van der Zalm et al., 2022). The US Environmental Protection Agency's (US EPA)
33 Endocrine Disruptor Screening Program has focused on identifying EDCs that interfere with the
34 estrogen, androgen, and thyroid systems (USEPA, 2022). Much work has been done in
35 validating *in vitro* assays to test chemical interactions at the estrogen and androgen receptors
36 to prevent reproductive impacts (Browne et al., 2015; Judson et al., 2015; Kleinstreuer et al.,
37 2017); however, progress in developing new methods for targets within the hypothalamus-
38 pituitary-thyroid (HPT) axis has been slower due to complexity of the thyroid system and
39 multiple targets for potential disruption (Noyes et al., 2019; USEPA, 2022).

40 The HPT axis is tightly regulated due to its importance in growth, development, and
41 metabolism (Boas et al., 2012; Nillni, 2010). There are several targets of potential interference
42 along this HPT axis as well as in serum binding proteins and peripheral tissues (Boas et al., 2012;
43 Boas et al., 2006; Tan & Zoeller, 2007; Zoeller et al., 2007). Further, a feedback system signaling
44 when to turn on and off production of thyroid hormone (TH) is in place to maintain TH

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

45 homeostasis (Costa-e-Sousa & Hollenberg, 2012; Hoermann et al., 2015). The complexity of this
46 system requires development of *in vitro* assays for each of the targets to get a comprehensive
47 view of what may be occuring *in vivo*. To date, several *in vitro* assays have been developed and
48 used to screen chemicals for interference in processes related to hypothalamic and pituitary
49 signaling, thyroid hormone signaling, and thyroid hormone synthesis. Specifically, the thyroid-
50 relevant assays used to screen the ToxCast chemical libraries include thyroid hormone receptor
51 (Freitas et al., 2011; Knudsen et al., 2011; Martin et al., 2010; Romanov et al., 2008), thyroid
52 stimulating hormone receptor (Murk et al., 2013; Titus et al., 2008), thyrotropin releasing
53 hormone receptor (Knudsen et al., 2011), thyroid peroxidase (Paul Friedman et al., 2016; Paul
54 et al., 2014), sodium iodide symporter (Hallinger et al., 2017; J. Wang et al., 2018),
55 iodothyronine deiodinases (Hornung et al., 2018; Mayasich et al., 2021; Olker et al., 2019;
56 Renko et al., 2012; Renko et al., 2015), and iodotyrosine deiodinase (Olker et al., 2021; Renko et
57 al., 2016). An area not currently covered by these assays are the thyroid hormone distributor
58 proteins.

59 Transthyretin (TTR), also called prealbumin, is one of the serum binding proteins
60 responsible for delivering thyroid hormones to target tissues and maintaining the balance of
61 free versus bound TH (Rabah et al., 2019; Richardson, 2007; Schreiber, 2002). TTR is
62 synthesized in the liver and in the choroid plexus for TH transport in plasma and cerebral spinal
63 fluid (Magalhaes et al., 2021; Rabah et al., 2019). TTR is suggested to mediate delivery of TH
64 across the blood-brain barrier and through the placenta (Meerts et al., 2002). TTR is a
65 homotetramer with two TH binding sites; however, due to negative cooperativity it is
66 understood to bind only one TH at a time (Richardson, 2002; Tomar et al., 2012; H. Wang et al.,

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

67 2007). In addition to TH, TTR can bind retinol binding protein, which in turn binds and
68 transports retinol (Prapunpoj & Leelawatwattana, 2009). Chemical binding to TTR can displace
69 TH from the protein resulting in lack of TH delivered to the target tissue and subsequent
70 disruption of biological pathways dependent on TH activation (Cao et al., 2011). Competitive
71 binding with TTR in cerebrospinal fluid has been shown *in vitro* to increase free T4 which may
72 negatively regulate production of TH (Radovic et al., 2006). Alternatively, TH freed from TTR
73 could be metabolized and excreted resulting in decreased TH levels in serum (Huang et al.,
74 2023; Kim et al., 2018). Assays have been developed to assess chemical binding to TTR. These
75 include a radiolabeled ligand displacement assay (Lans et al., 1993) and the fluorescence-based
76 competitive binding assays using fluorescein isothiocyanate-T4 conjugate (FITC-T4) or 8-anilino-
77 1-naphthalenesulfonic acid ammonium salt (ANSA) (Cao et al., 2010; Montaño et al., 2012; Ren
78 & Guo, 2012). The FITC and ANSA assays are currently being validated by the European Union
79 Reference Laboratory for Alternatives to Animal testing (EURL ECVAM,
80 tsar.jrc.ec.europa.eu/test-method/tm2019-08).

81 The objective of this work was to screen the ToxCast ph1_v2, ph2, and e1k libraries
82 (Richard et al., 2016) in the TTR-ANSA assay using a tiered approach to: 1) identify chemicals
83 with the potential to bind TTR, 2) determine an EC50 value for the chemicals that bind to TTR
84 and displace ANSA based on 85% activity or greater in single concentration screening, and 3)
85 evaluate the performance of the assay for use in large-scale screening.

86 Materials and Methods

87 *Overview*

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

88 Development of this TTR binding assay for use in our lab was based on methods
89 developed by Montaño et al. (2012) to identify chemicals that bind to the protein by measuring
90 a reduction in fluorescence due to displacement of ANSA. The assay was optimized in a 96-well
91 plate with a fluorescence readout that measures displacement of the probe from the protein.
92 The assay used human TTR, 8-anilino-1-naphthalene sulfonic acid (ANSA) as the fluorescent
93 probe, dimethyl sulfoxide (DMSO) as the negative control, and L-thyroxine (T4) as the positive
94 control. TTR, ANSA, DMSO, and T4 were purchased from Sigma-Aldrich (St Louis, MO). The
95 screening format followed Hornung et al. (2018), Olker et al. (2019), Olker et al. (2021), and
96 Degitz et al. (2023) with initial screening at a single concentration (target of 100 µM) followed
97 by concentration-response testing for a subset of chemicals. Most chemicals screened in
98 concentration-response were tested from 0.015 to 100 µM but overall concentrations ranged
99 from 0.004 to 150 µM due to chemicals being plated in source plates at different
100 concentrations (details in “Chemicals” section).

101 *Chemicals*

102 1813 unique chemicals from the ToxCast libraries (Richard et al., 2016) were obtained
103 from Evotec (Branford, CT) via the ToxCast program. The set included chemicals from the
104 ph1_v2, ph2, and e1k libraries. The chemicals were supplied across twenty-five 96-well
105 chemical source plates with one chemical per well. Quality control of these chemicals relied on
106 requirements of the ToxCast library [*e.g.*, identification, purity, analytical verification (Richard
107 et al., 2016)]. Chemical identities were masked until single concentration screening was
108 complete. Chemicals were provided at a target concentration of 20 mM in DMSO; however, for
109 some chemicals the plated concentration differed from the target concentration due to

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

110 solubility limitations in DMSO (120 chemicals <20 mM, 22 chemicals >20 mM). Further, the
111 concentration for 12 chemicals (oils and mixtures) was provided in mg/mL. Overall, 93.4% of
112 chemicals were provided within 1 mM of the target concentration.

113 In addition to test chemicals, DMSO, T4, and TTR were screened on each plate. DMSO,
114 the solvent control, served as a negative control due to its inability to displace ANSA from TTR.
115 T4 is a known ligand of TTR and served as a positive control. The final amount of DMSO in the
116 DMSO and T4 controls was 0.5% to match the amount added in test chemical reactions. A
117 protein-only control was included to correct fluorescence values. Source plates were received
118 with column 1 and several random wells left empty for addition of reference controls. A T4
119 standard curve was added in column 1 using a 10 mM stock solution prepared in 0.4 N NaOH
120 and diluted to final concentrations of 1.8, 0.81, 0.3645, 0.164, 0.0738, 0.0332. 0.0149, and
121 0.0067 μM. The maximum T4 concentration (1.8 μM) served as the positive control correlating
122 with complete displacement of ANSA from TTR. T4 concentrations were selected based on
123 assay optimization (see section below). Each replicate plate contained the T4 curve, additional
124 high T4 and low T4 wells, DMSO, and TTR. Plate layouts for single concentration screening and
125 concentration-response testing are in Supplemental Figure 1.

126 *Autofluorescence*

127 Reduction in fluorescence is used to determine displacement of ANSA from TTR; thus,
128 autofluorescence of test chemicals is a potential interference of this assay. Specifically, one
129 concern would be that autofluorescence of the chemical would mask any reduction in ANSA
130 fluorescence resulting in a false negative response. To address interference, 1 μL of each
131 chemical was added to 199 μL of phosphate buffer, incubated for 2 hours at 4°C, and

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

132 fluorescence was measured using a BioTek Synergy Neo2 plate reader (Agilent, Santa Clara, CA)
133 with an excitation wavelength of 380 nm and an emission wavelength of 475 nm.

134 *Binding assay optimization*

135 Several iterations of the binding assay were performed while optimizing this assay for
136 use in our lab. Initial testing followed the parameters outlined by Montaño et al. (2012) with
137 reactions containing 0.5 μM TTR, 0.6 μM ANSA, and a T4 curve ranging from 100-1000 nM.
138 Additionally, we assessed the difference in using clear vs black 96-well plates and flat vs round
139 bottom plates in an effort to minimize background fluorescence. Black round-bottom plates
140 (Corning 3356) were selected for use as assay plates. Further optimization included testing
141 different concentrations and ratios of TTR and ANSA with the goal of reducing the amount of
142 TTR in each reaction to ultimately lower the cost of the assay.

143 *Assay conditions*

144 Each 200 μL reaction contained 0.125 μM TTR, 1.2 μM ANSA, and 0.5% DMSO in 0.1 M
145 phosphate buffer, pH 7.5. Four individual master mixes were prepared: a main master mix for
146 test chemicals which contained all components except DMSO, the T4 and DMSO master mixes
147 which contained all components, and a TTR master mix which did not contain ANSA
148 (Supplemental Table 1). Master mixes were prepared in low light to prevent ANSA degredation.
149 Each master mix was loaded into the appropriate wells of a 2 mL 96-well reservoir plate. Using
150 a Biomek i5 liquid handler (Beckman Coulter, Indianapolis, IN), six 96-well black assay plates
151 were loaded with master mix. Subsequently, 2 μL T4 and 1 μL test chemical were added to the
152 assay plates using a 20 μL Rainin Liquidator 96-channel benchtop pipettor (Mettler Toledo,
153 Columbus, OH). Reactions were mixed by pipetting using a 200 μL Rainin Liquidator, sealed

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

154 with a polyolefin plate seal, and shaken on a Jitterbug microplate shaker (Boekel Scientific,
155 Feasterville, PA) for 2 minutes at 1000 rpm. Assay plates were covered with foil and incubated
156 at 4°C for 2 hours. Following incubation, fluorescence was measured using a BioTek Synergy
157 Neo2 plate reader (Agilent, Santa Clara, CA) using an excitation wavelength of 380 nm and an
158 emission wavelength of 475 nm.

159 *Chemical screening*

160 Chemical screening was performed using a tiered approach. The first tier was comprised
161 of screening each chemical at a single concentration. The target concentration for single
162 concentration screening was 100 µM with some variation based on solubility. Daughter plates
163 were thawed before use and were used to load three individual assay plates (n=3 for each
164 chemical, one well on each assay plate; see Supplemental figure 1A for plate layout).
165 Additionally, a "T4 source plate" was prepared. The T4 source plate contained an adequate
166 volume of each concentration of the T4 standard curve and the additional high and low T4
167 concentration wells. The T4 source plate was used to load individual assay plates using the
168 Liquidator as described previously. Data were normalized to percent of control with the low
169 concentration of T4 representing maximum fluorescence (no displacement of ANSA) and the
170 highest concentration of T4 representing no fluorescence (100% ANSA displaced). In single
171 concentration screening, chemicals were considered inactive if they displayed less than 20%
172 activity compared to the high T4 concentration control.

173 A subset of chemicals with the greatest activity moved forward to concentration-
174 response testing. Specifically, the 282 chemicals with 85% or greater median displacement of
175 ANSA were tested in 12-point concentration-response curves in an effort to establish EC50s for

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

176 these chemicals binding to TTR thereby displacing ANSA. The 85% activity level for moving
177 chemicals forward for concentration-response testing differed from previous thyroid screening
178 assays (*e.g.*, deiodinases used a 50% activity threshold). In this assay, a large proportion of
179 chemicals showed activity >50%; thus, selecting 85% activity as the threshold for moving
180 chemicals onto concentration-response testing provided better discrimination from a
181 prioritization standpoint. For concentration-response testing, chemicals were taken from the
182 original chemical source plate and aliquoted in a new 96-well polypropylene plate ("CR source
183 plate") to prepare dilutions in DMSO. Chemicals were tested in 12-point curves with target
184 concentrations of 0.015, 0.034, 0.076, 0.168, 0.374, 0.83, 1.845, 4.101, 9.113, 20.25, 45, and
185 100 µM, one chemical per row with different concentrations in each column tested in three
186 separate assay plates (n=3 for each chemical at each concentration, see Supplemental Figure 1B
187 for plate layout).

188 *Data Processing and Analysis*

189 Data were processed and analyzed in R [version 4.2.1,(R Core Team, 2022)] using an
190 automated pipeline that normalized data, calculated plate diagnostics, and assigned assay-
191 specific flags. Data were processed by: 1) adjusting relative fluorescence units (RFUs) by
192 subtracting fluorescence in the TTR-only wells, and 2) normalizing test chemical responses to
193 the concentration-response curve of the model chemical T4 fit to the Hill model with high
194 concentration T4 (1.8 µM) representing 100% of ANSA displacement (0% of control) and low T4
195 (0.0067 µM) representing 0% ANSA displacement (100% of control). Results are reported as
196 percent activity which was calculated as 100 minus the percent of control. Results for single

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 ORCID: https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. License: CC BY-NC 4.0

197 concentration screening are reported as percent activity calculated from the median of the
198 three replicates with range used to display variability.

199 Concentration-response data were analyzed using the ToxCast Analysis Pipeline (tcpl)
200 package version 3.0.1 (Filer, 2022) using the tcplLite option. In this analysis, percent activity was
201 used as the response value and 20% activity as the threshold cutoff. This 20% cutoff was
202 selected to have a consistent threshold across assays. The three replicates for each
203 concentration of a chemical were included to fit dose-response curves based on the constant,
204 constrained Hill, and constrained gain-loss model. The best model fit was identified based on
205 the lowest Akaike Information Criterion (AIC) value. For chemicals that fit the Hill or gain-loss
206 model, the EC20, EC50, and Hill slope were calculated from the model fit parameters. Results
207 from concentration-response screening are displayed as displacement from the maximum
208 response with chemical concentration (log10 scale) on the x-axis and percent of control on the
209 y-axis.

210 *Assay quality*

211 Quality control measures were calculated following methods previously described
212 (Olker et al., 2019; Olker et al., 2021). Briefly, variability and separation between the positive
213 control (1.8 µM T4) and solvent/negative control (DMSO) were evaluated with plate-wise Z'
214 factors. The Z' factor is an indicator of assay quality, and values above 0.5 indicate good
215 separation between positive and negative controls (Zhang et al., 1999). A Z' factor greater than
216 or equal to 0.5 was used as a guideline for acceptable plate runs. Typically assay plates were
217 rerun to replace data from plate runs where quality criteria were not met (*e.g.*, low Z' factor,

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

218 poor control data). In one instance, a Z' factor less than 0.5 was not re-run because the two
219 other replicates for that plate met the quality criteria.

220 Data that fell outside of acceptable parameters for variability and fluorescence were
221 flagged for manual review. Flags included high variability when the absolute difference
222 between the mean and median of three replicate runs was greater than 10% and potential
223 assay interference indicated by fluorescence outside of normal ranges. Individual wells with
224 observed problems (*e.g.*, outliers, low volume in a well in one replicate) were excluded from
225 analyses with only two replicates used.

226 **Results**

227 *Assay Optimization, Performance, Quality Control*

228 Optimization of the TTR binding assay in our lab resulted in a fluorescence-based assay
229 suitable for screening large chemical libraries for binding to the human TTR protein. To
230 evaluate data quality, standard curves, Z' factor, and variability of the controls were evaluated
231 for each assay plate and summarized across all plates. Quality control metrics were consistent
232 over the duration of data collection in triplicate testing of 25 single concentration plates and 52
233 concentration-response plates. Standard curves for the positive control (T4) were consistent
234 with mean EC50 of 0.075 ± 0.02 SD and mean Hill slope of -1.113 ± 0.29 SD. Standard curves for
235 all replicates had a Z' factor >0.5 except for one plate which failed acceptance criteria with a Z'
236 factor of 0.302. The low Z' factor in this plate was attributable to unusually low fluorescence in
237 a DMSO control well; however, the two other replicate plates had Z' factors >0.5, and this set of
238 plates was a repeat run with data similar to the initial assay run. Additionally, the mean Z'

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

239 factor across all plates was 0.883± 0.06 SD and ranged from 0.542 to 0.98 when the one
240 replicate plate was excluded.

241 *Autofluorescence*

242 The assessment of chemical autofluorescence was done after screening all chemicals in
243 the TTR assay to better understand an allowable threshold for chemical autofluorescence. For
244 this analysis, the threshold for allowable chemical autofluorescence was set at 10% of
245 maximum reaction fluorescence (*i.e.*, 10% of the average DMSO negative control fluorescence
246 across all single concentration and concentration-response plates). The average maximum
247 fluorescence in DMSO wells across all plates was 10263, so the threshold for chemical
248 autofluorescence was 1026.3. Based on this threshold, 262 chemicals were determined to be
249 autofluorescent (Figure 1). The 262 chemicals interfering with the assay are listed in
250 Supplemental Table S2. Additionally, 39 chemicals (Supplemental Table S3) were flagged during
251 data processing due to fluorescence being more than 20% greater than DMSO negative controls
252 (*i.e.*, RFUs > DMSO+20%). Due to these exclusions, results from 1512 of the 1813 chemicals are
253 reported herein.

254 *Single Concentration Screening*

255 Data from single concentration screening are being used in the Tox24 challenge for
256 independent researchers to develop methods to predict compounds' ability to bind TTR using
257 only chemical structure data (https://ochem.eu/static/challenge.do) (Tetko, 2024). Thus, results
258 from the blind and leaderboard sets are not included in this version of the manuscript but will
259 be made publicly available at the conclusion of the challenge. In single concentration screening,
260 624 chemicals had <20% activity compared to the activity of high concentration T4 controls and

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

261 were considered inactive (Table 1, Figure 2). Of the 888 chemicals with activity of 20% or
262 greater, 282 had 85% or greater activity and were moved onto concentration-response testing
263 (Supplemental Table S4).

264 *Concentration-response Testing*

265 Results from concentration-response testing confirmed single concentration screening
266 results for 96% of the 282 chemicals tested. Results were considered consistent when the
267 percent difference between median percent activity in single concentration screening and
268 median percent activity at the maximum concentration tested in concentration-response was
269 less than 25%. Two of the chemicals, *X* and tripropylene glycol monomethyl ether, were active
270 in single concentration screening and had less than 20% activity at the highest concentration
271 tested in concentration-response. Different sets of source plates were used for single
272 concentration and concentration-response testing; thus, it is possible that there was an error in
273 dosing in either of the assays. The remaining nine chemicals that were inconsistent ranged from
274 86-102% activity in single concentration screening and 32-73% activity at the maximum
275 concentration tested in concentration-response.

276 The Hill model was identified in the ToxCast pipeline as the best fit model for 230 of 282
277 chemicals, and the gain-loss model was the best fit model for 50 chemicals. EC50 values were
278 obtained for 276 of the chemicals screened in concentration-response (Supplemental Table 5,
279 Supplemental Figure 2). For the 6 chemicals with no EC50 reported, two were inactive at all
280 concentrations tested (*X* and tripropylene glycol monomethyl ether) and the constant model
281 was the winning fit. The other four chemicals (citronellol, *X*,

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

282 1,3,5,7−Tetramethyl−1,3,5,7−tetravinylcyclotetrasiloxane, and difenzoquat) did not reach 50%
283 activity at the highest concentration tested in concentration-response.

284 The 25 most potent chemicals, ranked by EC50, are listed in Table 2. Twenty-four of the
285 chemicals had EC50 values less than or equal to the mean EC50 of T4 indicating that these
286 chemicals are equally or more potent as T4 in binding to TTR. Example curves for selected
287 chemicals are displayed in Figure 2. Complete displacement of ANSA from TTR was not achieved
288 for the nine chemicals presented from the top twenty-five ranked inhibitors. These chemicals
289 were more (Figures 2B-2I) or equally (C.I. disperse Black 6 dihydrochloride, Figure 2J) potent
290 compared to T4 based on EC50. These chemicals could have been tested at even lower
291 concentrations to achieve 100% of control (zero activity) and establish a full concentration-
292 response curve.

293 **Discussion**

294 Investigating chemical binding to TTR is important due to TTR's role in transporting
295 thyroid hormone to target tissues as well as maintaining thyroid hormone homeostasis. It is
296 commonly understood that displacement of T4 from TTR results in enhanced clearance and
297 reduced serum TH concentrations (Brouwer et al., 1989; Morse et al., 1996; Yamauchi et al.,
298 2003). While some research has been done to investigate chemical binding to serum transport
299 proteins, this study presents the largest library of chemicals screened to date. Screening 1512
300 chemicals from the ToxCast chemical library through the TTR-ANSA binding assay presented
301 herein resulted in identification of 888 active chemicals (20% activity or greater). Of these, 282
302 produced 85% activity or greater including 243 chemicals that, to our knowledge, have not
303 previously been shown to bind TTR.

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

304 The data set was evaluated to determine whether any functional groups are enriched in
305 the highly active (>85% activity) chemicals compared to the less active chemicals screened in
306 this assay (Sushko et al., 2011; Vorberg & Tetko, 2014). SMILES were downloaded from the
307 CompTox Chemicals Dashboard for the ph1_v2, ph2, and e1k library lists. Following the
308 approach of Vorberg and Tetko (2014), SMILES were uploaded into the Online Chemical
309 Modeling Environment (OCHEM). SMILES were recognized for 1504 individual chemicals. The
310 chemicals were discretized by setting 85% activity as the threshold. There were 280 chemicals
311 recognized as having "high" activity (>85%), and the remaining chemicals were categorized as
312 "low" activity. The SetCompare Utility was then used to compare functional groups enriched
313 among the highly active chemicals (Table 4). This analysis revealed that azo compounds and
314 phenols are overrepresented in the highly active compounds with enrichment factors of 68 and
315 6.6, respectively. Interestingly, hydroxy compounds containing alcohols or phenols were
316 grouped together and found to be enriched among the highly active compounds; however, this
317 is largely due to enrichment of phenols (36.2% of high-actives vs 5.5% of low actives) since
318 alcohols are enriched in the low-active group (enrichment factor 4.8). The top 25 most active
319 chemicals (Table 2) were assessed for the presence of enriched functional groups. Most of the
320 25 most potent chemicals screened in this TTR assay are arenes, which were enriched by a
321 factor of 1.7 in highly active chemicals, and many of them are phenols. Further, thirteen of the
322 chemicals are halogen derivatives, which had an enrichment factor of 1.6. Three azo
323 compounds are present among the list of 25 most potent TTR binders.

324 Further investigation was done for the 25 most potent chemicals in this assay (lowest
325 EC50s) to determine whether they have demonstrated thyroid activity in other *in vitro* or *in vivo*

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

326 assays. Specifically, *in vitro* activity was assessed in thyroid assays with data available through
327 the US EPA's CompTox Chemicals Dashboard (USEPA, 2021). The majority of the top 25 potent
328 chemicals are active in multiple thyroid relevant assays. All of the chemicals except for 2,4,6-
329 trichlorophenol were active in at least one other *in vitro* assay. Three chemicals (bromoxynil,
330 chlorthal-dimethyl, and ammonium perfluorooctanoate) were active in only one other *in vitro*
331 assay, which was the Tox21_TR_Luc_GH3_antagonist assay. Twenty-three of the twenty-five
332 chemicals were active in the Tox21_TR_Luc_GH3_antagonist assay; however, eighteen of them
333 were also active in the associated viability assay indicating potential cytotoxicity. It has been
334 reported that the Tox21_TR_Luc_GH3 antagonist assay is sensitive and identifies chemicals as
335 active even if they are not direct thyroid receptor antagonists (Paul-Friedman et al., 2019).
336 Chemical activity in the TTR binding assay and in other assays in which TH is the substrate
337 (iodothyronine deiodinases) or ligand (thyroid receptor) makes sense due to TH being
338 endogenous ligands for TTR. However, several chemicals are also active in the thyroid
339 peroxidase (stimulated by thyroid-stimulating hormone) and iodotyrosine deiodinase (recycles
340 iodide from mono- and diiodotyrosine) assays which are not regulated by TH, suggesting that
341 these chemicals have non-specific interactions with multiple targets in the thyroid axis. To
342 further clarify thyroid activity of these chemicals, a basic review of the literature was performed
343 to identify *in vivo* thyroid activity. The review was performed by searching "(thyroid OR T4) AND
344 (chemical name)" in Web of Science (search conducted July 24, 2023). As anticipated, there are
345 many reports of *in vivo* thyroid activity for the thyroid hormone analogs Tetrac (ranked 1) and
346 Triac (ranked 4). No *in vivo* studies were found for eighteen of the chemicals. Thyroid activity
347 was demonstrated *in vivo* for the remaining five chemicals: ammonium perfluorooctanoate

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

348 (Butenhoff et al., 2012), 2,4,6-tribromophenol (Fu et al., 2020; Lee et al., 2016),
349 diethylstilbestrol (Lin et al., 2023), apigenin (Panda & Kar, 2007), and dinoseb (Van den Berg et
350 al., 1991).

351 The EC50 values generated in this study were compared with those from previous
352 screening efforts using radiolabeled ligand (^{125}I-T4) as well as fluorescein isothiocyanate (FITC-
353 T4) and 8-anilino-1-napthalene sulfonic acid (ANSA) assays (Table 4). There was a total of thirty-
354 six chemicals overlapping between previous TTR screening assays and the ToxCast libraries.
355 There are some chemicals (*e.g.*, amiodarone, benzoic acid, methimazole) with reported EC50s
356 from previous ANSA screening assays greater than 24 µM which was corroborated by the ANSA
357 assay reported herein by low percent activity in single concentration screening which precluded
358 the chemicals from being screened in concentration-response. Similarly, several chemicals
359 screened in the ^{125}I-T4 assay had indeterminable EC50 values (2-perfluorohexyl ethanol,
360 bisphenol A diglycidyl ether, hexanoic acid, and octanoic acid) which were not screened in
361 concentration-response in this study due to low activity in single concentration screening.
362 Interestingly, bisphenol A had 96% activity in single concentration screening but a relatively
363 high EC50 value of 8.63 µM. The EC50 for bisphenol A was not determined in a previous ^{125}I-T4
364 assay suggesting this chemical does not have a particularly high affinity for TTR which agrees
365 with the higher EC50 value determined in this study. In general, the ANSA-TTR assay conditions
366 in this study appear to be more sensitive than other TTR binding assays with amiodarone in the
367 FITC assay as the only exception. It has been reported that TTR is sensitive to changes in assay
368 conditions (Chauhan et al., 2000; Marchesini et al., 2006); thus, the variety of methods and
369 assay protocols used to investigate chemical binding to TTR hinder quantitative comparisons

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

370 (Marchesini et al., 2006). However, the T4 EC50 values reported in this study are in the range of
371 those reported in literature (Table 3).

372 There are costs and benefits to each of the three screening methods previously used.
373 The radio-labeled method uses the endogenous ligand of TTR to assess competition of
374 chemicals for binding to the protein; however, the radiolabeled substrate can be expensive and
375 hazardous, and the assay is not easy to adapt to a medium or high throughput format. ANSA is
376 relatively inexpensive and non-toxic, but it is known to bind to many proteins by nonselective
377 hydrophobic forces and cannot be used to specifically explore ligand binding sites (Ren & Guo,
378 2012). FITC may be more sensitive than ANSA due to the fluorescent probe being conjugated to
379 the endogenous ligand; however, the FITC assay may be less sensitive than the radiolabeled
380 ligand binding assay due to steric hindrance of the FITC moiety reducing the binding affinity of
381 the probe (Ouyang et al., 2017).

382 Based on assay performance metrics and the high Z' factors over the duration of
383 screening, this ANSA fluorescence-based assay is suitable for high throughput screening of
384 chemicals for binding to TTR; however, the large percentage (58.7%) of active chemicals may
385 indicate that the assay may not be sufficiently specific for use in risk assessment. It is also
386 possible that the TTR homotetrameric structure is not maintained *in vitro* and the reduction in
387 fluorescence for the presumably active chemicals may be a result of protein degradation as
388 opposed to chemical binding; however, this was previously tested in our lab with several per-
389 and polyfluoroakyl substances and degradation of the protein structure was not observed. This
390 supports the need for development of orthogonal high-throughput screening assays to confirm
391 these chemicals are truly active. Overall, results from this assay, especially in conjunction with

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

392 additional thyroid relevant assays, may be used to identify chemicals to prioritize for higher tier
393 testing.

394 **Disclaimer**

395 The views expressed in this paper are those of the authors and do not necessarily reflect the
396 views or policies of the U.S. Environmental Protection Agency. Mention of trade names or
397 commercial products does not constitute endorsement or recommendation for use.

398 **Funding**

399 This work was supported by the US Environmental Protection Agency.

400 **Acknowledgements**

401 The authors thank Katherine Coutros (US EPA, Center for Computational Toxicology and
402 Exposure) for assistance obtaining the ToxCast chemical libraries for screening. The authors
403 thank Dr. Tammy Stoker (U.S. EPA, Center for Public Health and Environmental Assessment)
404 and Dr. Dan Villeneuve (U.S. EPA, Center for Computational Toxicology and Exposure) for
405 providing comments on an early draft of the manuscript. This project was supported in part by
406 an appointment to the Research Participation Program at the Office of Research and
407 Development Center for Computational Toxicology and Exposure, U.S. Environmental
408 Protection Agency, administered by the Oak Ridge Institute for Science and Education through
409 an interagency agreement between the U.S. Department of Energy and EPA.

410

411

412

413

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

414 **Tables**

415 **Table 1.** Summary of single point screening at an initial target concentration of 100 µM: percent
416 and number of chemicals in each library that produced less than 20% or greater than/equal to
417 20%, 50%, and 85% activity. ***Actual % and # withheld until the end of the Tox24 Challenge**
418

Chemical library	# chemicals tested[a]	% with < 20% activity (#)	% with ≥ 20% activity (#)	% with ≥ 50% activity (#)	% with ≥ 85% activity (#)
ToxCast ph1_v2	262	% (#)	% (#)	% (#)	% (#)
ToxCast ph2	578	% (#)	% (#)	% (#)	% (#)
ToxCast e1k	672	% (#)	% (#)	% (#)	% (#)
Total	1512	% (#)	% (#)	% (#)	% (#)

419
420 Chemical names, CAS registry numbers, maximum tested concentrations, and median % activity produced can be
421 found in Supplementary Table S3.
422
423 [a]Chemical plates received from ToxCast included 1813 unique chemicals; however, 301 compounds had evidence
424 of interfering with the TTR assay and were excluded from all summaries and analyses.
425
426

427

428

429

430

431

432

433

434

435

436

437

438

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

439 **Table 2.** The top 25 ranked chemicals for binding to transthyretin (TTR) based on EC50.

440 ***Chemicals from the blind and leaderboard sets withheld until the end of the Tox24 challenge.***

Rank	Chemical	CASRN	Max Tested Conc., µM	% Activity at Max Conc.[a]	EC20 (µM)	EC50 (µM)
1	Tetrac	67-30-1	100	107.7	0.011	0.017
2	o-Aminoazotoluene	97-56-3	100	109.4	0.007	0.018
3	4,6-Dinitro-o-cresol	534-52-1	100	109.5	0.004	0.020
4	Tiratricol	51-24-1	100	104.7	0.008	0.021
5	Bromoxynil	1689-84-5	80	103.5	0.007	0.022
6	3,3',5,5'-Tetrabromobisphenol A	79-94-7	100	107.2	0.015	0.024
7	Diethylstilbestrol	56-53-1	100	104.3	0.011	0.025
8	2,4,6-Tribromophenol	118-79-6	100	106.3	0.014	0.028
9	Acid Red 337	67786-14-5	100	107.0	0.013	0.033
10	2,2',6,6'-Tetrachlorobisphenol A	79-95-8	100	106.9	0.012	0.033
11	2-Chloro-4-phenylphenol	92-04-6	100	106.1	0.015	0.034
12	4-Bromo-2-(4-chlorophenyl)-1-(ethoxymethyl)-5-(trifluoromethyl)-1H-pyrrole-3-carbonitrile	122453-73-0	100	103.1	0.017	0.035
13	Apigenin	520-36-5	100	104.4	0.012	0.035
14	Dinocap	39300-45-3	100	112.0	0.012	0.036
15	2,4,6-Trichlorophenol	88-06-2	100	103.9	0.011	0.042
16						
17	2,4-Dihydroxybenzophenone	131-56-6	100	105.5	0.010	0.045
18	Phenolphthalein	77-09-8	100	104.5	0.007	0.049
19	Chlorthal-dimethyl	1861-32-1	100	103.2	0.012	0.055
20	Chrysin	480-40-0	100	105.3	0.013	0.055
21	Fluazinam	79622-59-6	100	109.8	0.016	0.062
22						
	L-thyroxine (T4, positive control)	**51-48-9**	**1.8**			**0.075 ±0.02**
23	Ammonium perfluorooctanoate	3825-26-1	100	103.8	0.011	0.075
24	C.I. Disperse Black 6 dihydrochloride	20325-40-0	100	111.4	0.018	0.080

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

25	Dinoseb	88-85-7	50	110.6	0.19	0.081

441 [a]Median of three replicates at maximum concentration of chemical in concentration-response testing.

442

443

444

445

446

447

448

449

450

451

452

453

454

455

456

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 ORCID: https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. License: CC BY-NC 4.0

457 **Table 3.** Analysis of functional groups enriched in high and low TTR binding activity groups using
458 the SetCompare Utility of OCHEM.
459

Descriptor	High activity		Low activity		Enrichment factor	p value
	#	%	#	%		
Phenols	101	36.2	66	5.5	6.6	3.86E-38
Arenes	217	77.8	555	46	1.7	8.27E-23
Aromatic compounds	225	80.6	624	51.7	1.6	6.05E-20
Azo	19	6.8	1	0.1	68	1.57E-13
Hydroxy compounds: alcohols or phenols	110	39.4	258	21.4	1.8	1.02E-09
Alcohols	11	3.9	196	16.2	4.2	-2.19E-09
Sulfonic acid derivatives	34	12.2	50	4.1	3	1.54E-06
Sulfonic acids	20	7.2	18	1.5	4.8	2.04E-06
Aryl halides	71	25.4	164	13.6	1.9	2.29E-06
Halogen derivatives (alkyl, alkenyl, aryl)	99	35.5	268	22.2	1.6	4.68E-06

460
461
462
463
464
465
466
467
468
469
470
471
472

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

473 **Table 4.** Comparison of EC50 values from literature and from this study. Assays include the TTR-
474 radiolabeled ligand binding assay (^{125}I-T4) as well as the fluorescein-5-isothiocyante (FITC-T4) and 8-
475 anilino-1-naphthalene sulfonic acid (ANSA) fluorescent binding assays. Percent activity from single point
476 screening in this study is also reported to explain why some chemicals do not have a reported EC50 (e.g.,
477 <85% activity in SC was not run in concentration response). ***Chemicals from the blind and**
478 ***leaderboard sets withheld until the end of the Tox24 challenge.***
479

	Previous TTR screening*			This study	
Chemical	^{125}I-T4 EC50 (µM)	FITC-T4 EC50 (µM)	ANSA EC50 (µM)	ANSA EC50 (µM)	% Activity in Single conc.
T4	0.049[2] 0.055[4] 0.091[6] 0.088[7] 0.087[9] 0.08[12] 0.061[15]	0.1[5] 0.057[8] 0.26[11] 0.26[13] 0.031[14]	0.03[1] 0.21[3] 0.42[8] 0.26[10] 0.685[16] 0.685[18]	0.075	
Tetraiodothyroacetic acid (Tetrac)		0.029[8]	0.28[8]	0.016	105
Triiodothyroacetic acid (Triac, Tiratricol)		0.020[8]	0.28[8]	0.021	105
2,4,6-tribromophenol	0.0048[4] 0.0672[9]	0.5[11]	0.37[18]	0.028	105
2,4,6-trichlorophenol			1.41[18]	0.04	100
2-perfluorohexyl ethanol	ND[15]			NA	56.8
4-nonylphenol		16[5]		3	89.2
Amiodarone		1.9[8]	>24[8]	NA	48.4
Benzoic acid			>1000[16]	NA	-17.1
Bisphenol A	ND[9]			8.63	96
Bisphenol A diglycidyl ether	ND[9]			NA	39.5
Decanoic acid	ND[15]			7.4	93.7
Fluorotelomer alcohol 8:2		ND[14]		NA	45.8
Hexanoic acid	ND[15]			NA	11.2
Linoleic acid			6.5[10]	NA	74.5
Mono(2-ethylhexyl)phthalate (MEHP)		13.08[11]	31.55[11]	1.34	99
Methimazole		>10[8]	>24[8]	NA	60.3
N-ethyl perfluorooctane sulfonamide	ND[15]			1.0	94.7
Octanoic acid	ND[15]			NA	80.5
Oleic acid			3.3[10]	3.0	98.2
Perfluorobutane sulfonate	19.46[15]†	13.331[16]†		1.76	99.4
Perfluorodecanoic acid	8.954[15]	3.1[5] 1.623[14]	0.71[3]	0.65	102.9
Perfluoroheptanoic acid	1.565[15]	1.2[5] 1.128[14]	0.4[3]	0.21	102.2
Perfluorohexane sulfonate	0.717[15]	0.594[14]†		0.09	102.4

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

Perfluorohexanoic acid	8.22[15]	5.6[5] 3.189[14]	1.4[3]	1.05	96.4
Perfluorononanoic acid	2.737[15]	1.1[5] 1.977[14]		0.2	104.7
Perfluorooctane sulfonamide	6.124[15]	0.73[5]		0.61	103.1
Perfluorooctane sulfonate	0.94[15]	0.13[14]† 2.1[17]†		0.13	104.2
Perfluorooctane sulfonic acid		0.25[5] 1.21[11]		0.09	104.9
Perfluorooctanoic acid	0.949[15]	1.1[5] 2.02[11] 0.378[14]		0.15	103.5
Perfluoroundecanoic acid	21.56[15]	3.1[5] 5.339[14]	1.99[3]	0.69	101.4
Propylparaben		19.0[5]		5.0	96.8
Tetrabromobisphenol A	0.031[4] 0.0077[9]	0.034[5] 0.22[11]		0.02	105.8
Tetrachlorobisphenol A	0.1068[9]			0.03	105.2
Triclosan		1.1[5] 0.93[11]		0.16	106

*Chemicals that bind TTR in the literature are often referred to as "TTR inhibitors" because they potentially inhibit TH from binding TTR. As such, results from TTR assays in found in the literature are often reported as IC50s compared to EC50s reported in this study. For clarity, all the values will be referred to as EC50s in this discussion.

[1]Cao 2010, [2]Chauhan 2000, [3]Degitz in review, [4]Hamers 2006, [5]Hamers 2020, [6]Hill 2017 [7]Lans 1993, [8]Leusch 2018, [9]Meerts 2000, [10]Montaño 2012, [11]Ouyang 2017, [12]Radovic 2006, [13]Ren and Guo 2012, [14]Ren 2016, [15]Weiss 2009, [16]Xi 2020, [17]Xin 2018, [18]Yang 2019. †May be different formulation from what was screened in this study; no CAS was provided in the reference literature for comparison. These chemicals in the ToxCast libraries were provided as potassium salt.

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

497 **Figures and Figure Legends**

498 **Figure 1.** Fluorescence of chemicals from the ToxCast phase 1_v2, phase 2, and e1k libraries at
499 a target concentration of 100 uM. Chemicals are ordered from low to high according to the
500 average relative fluorescence units (RFUs) of three replicate plates. The dashed line denotes
501 10% of the maximum fluorescence value from DMSO wells across all plates, which was the
502 maximum chemical autofluorescence set for this study. Chemicals above the dashed line (i.e.,
503 those with RFUs >1026) were excluded from further analysis. There were 68 additional
504 chemicals not depicted on the plot due to RFUs ranging from 10,200 to "overflow" (≥100,000).
505

506 **Figure 2.** Displacement of ANSA from transthyretin (TTR) by chemicals in single-concentration
507 screening of the ToxCast phase 1_v2, phase 2, and e1k libraries at a target concentration of 100
508 uM. Results are displayed as percent of control with median (black square) and range (line) of
509 three replicates. (A) All chemicals, (B) Expanded plot of chemicals with ≥85% displacement
510 (≤15% of control; displacement is 100-percent of control). Chemicals are plotted by rank order
511 based on median % of control from low to high. Chemicals further tested in concentration-
512 response screening are those below the orange dashed line which denotes 85% displacement.
513

514 **Figure 3.** Concentration-response curves for binding to transthyretin by T4 at eight
515 concentrations of the model ligand T4 (A) and selected chemicals at twelve concentrations with
516 three replicates at each concentration (black circles). Examples include nine of the top twenty-
517 five ranked inhibitors (B-J) selected from Table 2 and two chemicals with ~90% activity in single
518 concentration screening (K-L) to represent a variety across types of compounds and inhibition
519 curves.
520

521

522

523

524

525

526

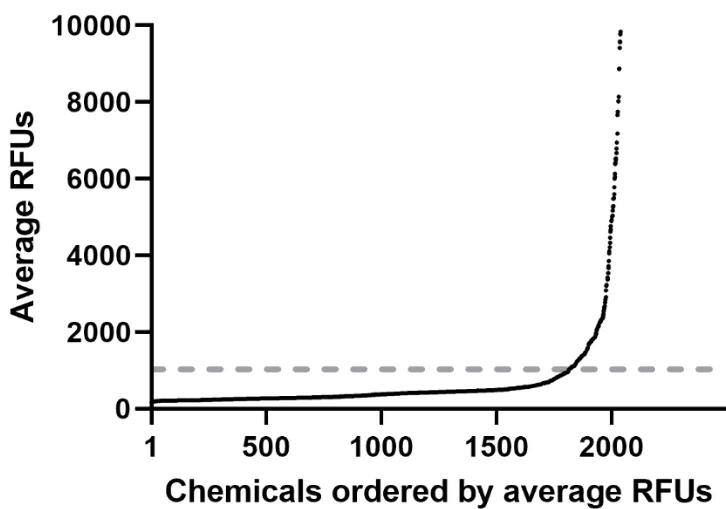
527

528

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

529 **Figure 1**

530



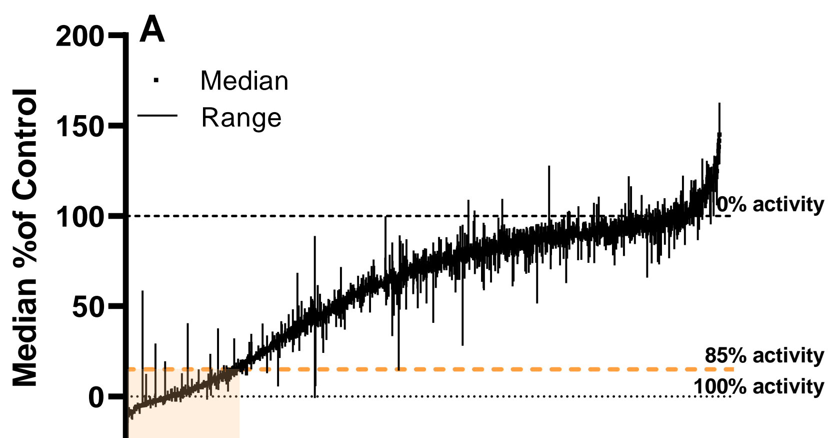
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

551 **Figure 2**

552

553



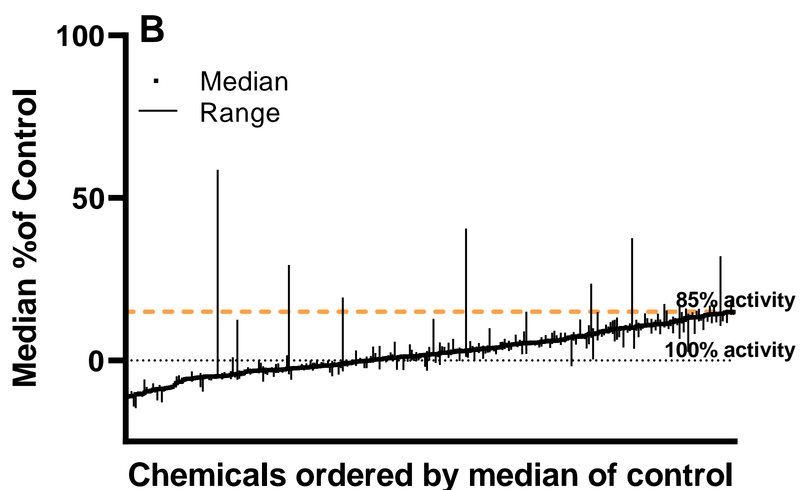
554

555

556

557

558



559

560

561

562

563

564

565

566

567

568

569

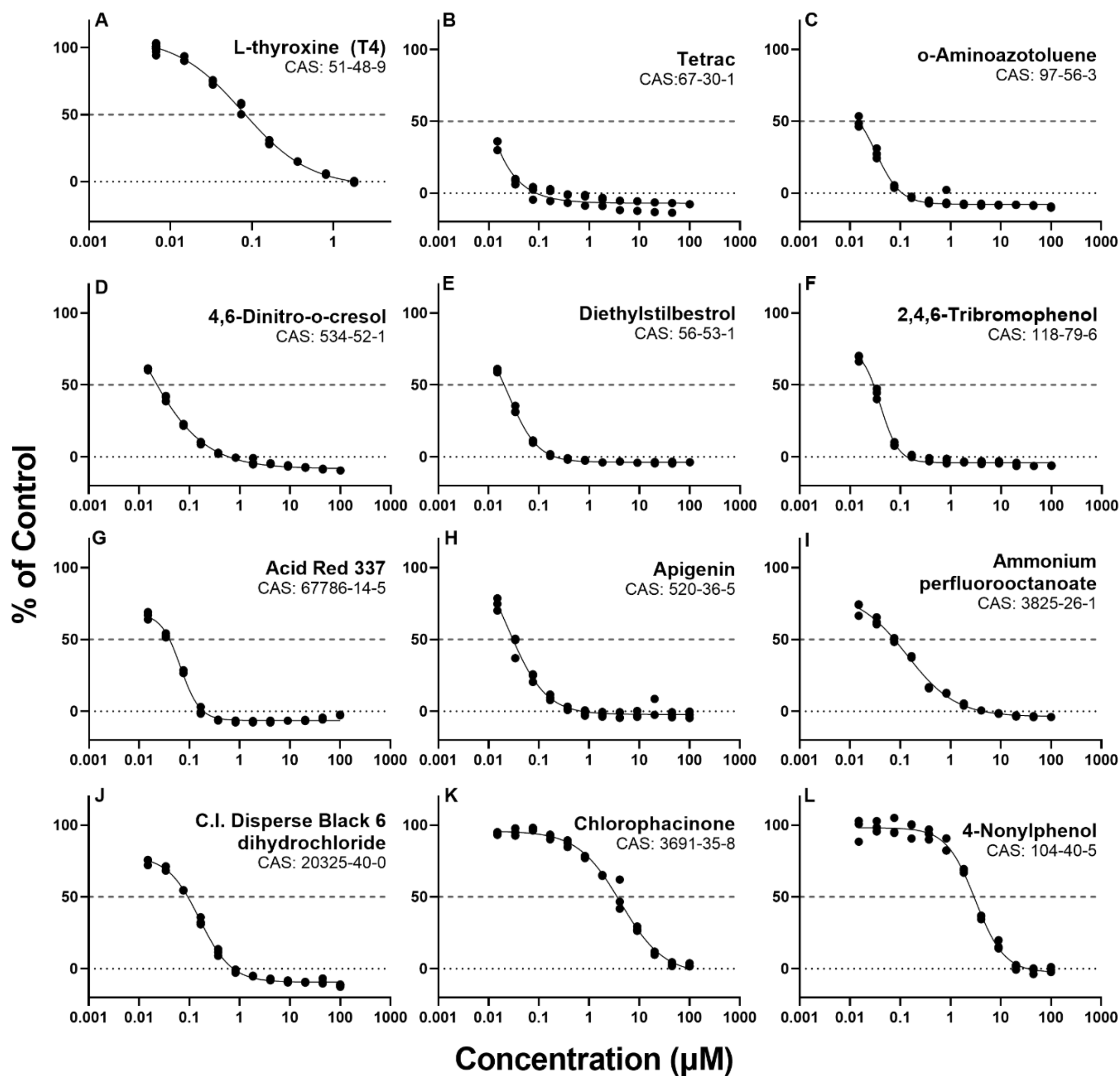
570

571

572

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

573 **Figure 3**



574

575

576

577

578

579

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

580 **References**

581 Boas, M., Feldt-Rasmussen, U., & Main, K. M. (2012). Thyroid effects of endocrine disrupting chemicals.
582 *Mol Cell Endocrinol, 355*(2), 240-248. doi:10.1016/j.mce.2011.09.005
583 Boas, M., Feldt-Rasmussen, U., Skakkebaek, N. E., & Main, K. M. (2006). Environmental chemicals and
584 thyroid function. *Eur J Endocrinol, 154*(5), 599-611. doi:10.1530/eje.1.02128
585 Brouwer, A., Reijnders, P. J. H., & Koeman, J. H. (1989). Polychlorinated biphenyl (PCB)-contaminated
586 fish induces vitamin A and thyroid hormone deficiency in the common seal (Phoca vitulina).
587 *Aquatic Toxicology, 15*(1), 99-106. doi:Doi 10.1016/0166-445x(89)90008-8
588 Browne, P., Judson, R. S., Casey, W. M., Kleinstreuer, N. C., & Thomas, R. S. (2015). Screening Chemicals
589 for Estrogen Receptor Bioactivity Using a Computational Model. *Environ Sci Technol, 49*(14),
590 8804-8814. doi:10.1021/acs.est.5b02641
591 Butenhoff, J. L., Bjork, J. A., Chang, S. C., Ehresman, D. J., Parker, G. A., Das, K., . . . Wallace, K. B. (2012).
592 Toxicological evaluation of ammonium perfluorobutyrate in rats: twenty-eight-day and ninety-
593 day oral gavage studies. *Reprod Toxicol, 33*(4), 513-530. doi:10.1016/j.reprotox.2011.08.004
594 Cao, J., Guo, L. H., Wan, B., & Wei, Y. (2011). In vitro fluorescence displacement investigation of
595 thyroxine transport disruption by bisphenol A. *J Environ Sci (China), 23*(2), 315-321.
596 doi:10.1016/s1001-0742(10)60408-1
597 Cao, J., Lin, Y., Guo, L. H., Zhang, A. Q., Wei, Y., & Yang, Y. (2010). Structure-based investigation on the
598 binding interaction of hydroxylated polybrominated diphenyl ethers with thyroxine transport
599 proteins. *Toxicology, 277*(1-3), 20-28. doi:10.1016/j.tox.2010.08.012
600 Chauhan, K. R., Kodavanti, P. R., & McKinney, J. D. (2000). Assessing the role of ortho-substitution on
601 polychlorinated biphenyl binding to transthyretin, a thyroxine transport protein. *Toxicol Appl*
602 *Pharmacol, 162*(1), 10-21. doi:10.1006/taap.1999.8826
603 Costa-e-Sousa, R. H., & Hollenberg, A. N. (2012). Minireview: The neural regulation of the hypothalamic-
604 pituitary-thyroid axis. *Endocrinology, 153*(9), 4128-4135. doi:10.1210/en.2012-1467
605 Degitz, S. J., Olker, J. H., Denny, J. S., Degoey, P. P., Hartig, P. C., Cardon, M. C., . . . Hornung, M. W.
606 (2023). In vitro screening of per- and polyfluorinated substances (PFAS) for interference with
607 seven thyroid hormone system targets across nine assays. *Toxicol In Vitro, 95*, 105762.
608 doi:10.1016/j.tiv.2023.105762
609 ECVAM, E. TSAR-Tracking System for Alternative methods towards Regulatory acceptance. Retrieved
610 from https://tsar.jrc.ec.europa.eu/index.php/
611 Filer, D. (2022). _tcpl: ToxCast Data Analysis Pipeline_ (Version R package version 3.0.1). Retrieved from
612 https://CRAN.R-project.org/package=tcpl
613 Freitas, J., Cano, P., Craig-Veit, C., Goodson, M. L., Furlow, J. D., & Murk, A. J. (2011). Detection of
614 thyroid hormone receptor disruptors by a novel stable in vitro reporter gene assay. *Toxicol In*
615 *Vitro, 25*(1), 257-266. doi:10.1016/j.tiv.2010.08.013
616 Fu, J., Guo, Y., Wang, M., Yang, L., Han, J., Lee, J. S., & Zhou, B. (2020). Bioconcentration of 2,4,6-
617 tribromophenol (TBP) and thyroid endocrine disruption in zebrafish larvae. *Ecotoxicol Environ*
618 *Saf, 206*, 111207. doi:10.1016/j.ecoenv.2020.111207
619 Hallinger, D. R., Murr, A. S., Buckalew, A. R., Simmons, S. O., Stoker, T. E., & Laws, S. C. (2017).
620 Development of a screening approach to detect thyroid disrupting chemicals that inhibit the
621 human sodium iodide symporter (NIS). *Toxicol In Vitro, 40*, 66-78. doi:10.1016/j.tiv.2016.12.006
622 Hoermann, R., Midgley, J. E., Larisch, R., & Dietrich, J. W. (2015). Homeostatic Control of the Thyroid-
623 Pituitary Axis: Perspectives for Diagnosis and Treatment. *Front Endocrinol (Lausanne), 6*, 177.
624 doi:10.3389/fendo.2015.00177

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

625 Hornung, M. W., Korte, J. J., Olker, J. H., Denny, J. S., Knutsen, C., Hartig, P. C., . . . Degitz, S. J. (2018).
626 Screening the ToxCast Phase 1 Chemical Library for Inhibition of Deiodinase Type 1 Activity.
627 *Toxicol Sci, 162*(2), 570-581. doi:10.1093/toxsci/kfx279
628 Huang, K., Zhou, W., Fu, J., Zhang, Q., Teng, Y., Gu, L., . . . Jiang, G. (2023). Linking Transthyretin-Binding
629 Chemicals and Free Thyroid Hormones: In Vitro to In Vivo Extrapolation Based on a Competitive
630 Binding Model. *Environ Sci Technol, 57*(25), 9130-9139. doi:10.1021/acs.est.3c01094
631 Judson, R. S., Magpantay, F. M., Chickarmane, V., Haskell, C., Tania, N., Taylor, J., . . . Thomas, R. S.
632 (2015). Integrated Model of Chemical Perturbations of a Biological Pathway Using 18 In Vitro
633 High-Throughput Screening Assays for the Estrogen Receptor. *Toxicol Sci, 148*(1), 137-154.
634 doi:10.1093/toxsci/kfv168
635 Kim, M. J., Moon, S., Oh, B. C., Jung, D., Ji, K., Choi, K., & Park, Y. J. (2018). Association between
636 perfluoroalkyl substances exposure and thyroid function in adults: A meta-analysis. *PLoS One,*
637 *13*(5), e0197244. doi:10.1371/journal.pone.0197244
638 Kleinstreuer, N. C., Ceger, P., Watt, E. D., Martin, M., Houck, K., Browne, P., . . . Judson, R. (2017).
639 Development and Validation of a Computational Model for Androgen Receptor Activity. *Chem*
640 *Res Toxicol, 30*(4), 946-964. doi:10.1021/acs.chemrestox.6b00347
641 Knudsen, T. B., Houck, K. A., Sipes, N. S., Singh, A. V., Judson, R. S., Martin, M. T., . . . Kavlock, R. J. (2011).
642 Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets. *Toxicology,*
643 *282*(1-2), 1-15. doi:10.1016/j.tox.2010.12.010
644 Lans, M. C., Klasson-Wehler, E., Willemsen, M., Meussen, E., Safe, S., & Bouwer, A. (1993). Structure-
645 Dependent, Competetive interactin of hydroxy-polychlorobiphenyls, -dibenzo-p-dioxins, and -
646 dibenzofurans with human transthyretin. *Chem. Biol. Interactions, 88*, 7-21.
647 Lazofsky, A., & Buckley, B. (2022). Recent Trends in Multiclass Analysis of Emerging Endocrine Disrupting
648 Contaminants (EDCs) in Drinking Water. *Molecules, 27*(24). doi:10.3390/molecules27248835
649 Lee, D., Ahn, C., Hong, E. J., An, B. S., Hyun, S. H., Choi, K. C., & Jeung, E. B. (2016). 2,4,6-Tribromophenol
650 Interferes with the Thyroid Hormone System by Regulating Thyroid Hormones and the
651 Responsible Genes in Mice. *Int J Environ Res Public Health, 13*(7). doi:10.3390/ijerph13070697
652 Lin, X., Wang, Y., Yang, X., Watson, P., Yang, F., & Liu, H. (2023). Endocrine disrupting effect and
653 reproductive toxicity of the separate exposure and co-exposure of nano-polystyrene and
654 diethylstilbestrol to zebrafish. *Sci Total Environ, 865*, 161100.
655 doi:10.1016/j.scitotenv.2022.161100
656 Magalhaes, J., Eira, J., & Liz, M. A. (2021). The role of transthyretin in cell biology: impact on human
657 pathophysiology. *Cell Mol Life Sci, 78*(17-18), 6105-6117. doi:10.1007/s00018-021-03899-3
658 Marchesini, G. R., Meulenberg, E., Haasnoot, W., Mizuguchi, M., & Irth, H. (2006). Biosensor recognition
659 of thyroid-disrupting chemicals using transport proteins. *Anal Chem, 78*(4), 1107-1114.
660 doi:10.1021/ac051399i
661 Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., . . . Houck, K. A. (2010).
662 Impact of environmental chemicals on key transcription regulators and correlation to toxicity
663 end points within EPA's ToxCast program. *Chem Res Toxicol, 23*(3), 578-590.
664 doi:10.1021/tx900325g
665 Mayasich, S. A., Korte, J. J., Denny, J. S., Hartig, P. C., Olker, J. H., DeGoey, P., . . . Hornung, M. W. (2021).
666 Xenopus laevis and human type 3 iodothyronine deiodinase enzyme cross-species sensitivity to
667 inhibition by ToxCast chemicals. *Toxicol In Vitro, 73*, 105141. doi:10.1016/j.tiv.2021.105141
668 Meerts, I. A. T. M., Assink, Y., Cenijn, P. H., van den Berg, J. H. J., Weijers, B. M., Bergman, A., . . .
669 Brouwer, A. (2002). Placental Transfer of a Hydroxylated Polychlorinated Biphenyl and Effects on
670 Fetal and Maternal Thyroid Hormone Homeostasis in the Rat. *Toxicological Sciences, 68*, 361-
671 371.

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

672 Mondou, M., Hickey, G. M., Rahman, H. T., Maguire, S., Pain, G., Crump, D., . . . Basu, N. (2020). Factors
673 Affecting the Perception of New Approach Methodologies (NAMs) in the Ecotoxicology
674 Community. *Integr Environ Assess Manag, 16*(2), 269-281. doi:10.1002/ieam.4244
675 Montaño, M., Cocco, E., Guignard, C., Marsh, G., Hoffmann, L., Bergman, A., . . . Murk, A. J. (2012). New
676 approaches to assess the transthyretin binding capacity of bioactivated thyroid hormone
677 disruptors. *Toxicol Sci, 130*(1), 94-105. doi:10.1093/toxsci/kfs228
678 Morse, D. C., Klasson Wehler, E., Wesseling, W., Koeman, J. H., & Brouwer, A. (1996). Alterations in Rat
679 Brain Thyroid Hormone Status Following Pre- and Postnatal Exposure to Polychlorinated
680 Biphenyls (Aroclor 1254). *Toxicology and Applied Pharmacology, 136*(2), 269-279.
681 doi:10.1006/taap.1996.0034
682 Murk, A. J., Rijntjes, E., Blaauboer, B. J., Clewell, R., Crofton, K. M., Dingemans, M. M., . . . Gutleb, A. C.
683 (2013). Mechanism-based testing strategy using in vitro approaches for identification of thyroid
684 hormone disrupting chemicals. *Toxicol In Vitro, 27*(4), 1320-1346. doi:10.1016/j.tiv.2013.02.012
685 Naidu, R., Biswas, B., Willett, I. R., Cribb, J., Kumar Singh, B., Paul Nathanail, C., . . . Aitken, R. J. (2021).
686 Chemical pollution: A growing peril and potential catastrophic risk to humanity. *Environ Int, 156*,
687 106616. doi:10.1016/j.envint.2021.106616
688 Nillni, E. A. (2010). Regulation of the hypothalamic thyrotropin releasing hormone (TRH) neuron by
689 neuronal and peripheral inputs. *Front Neuroendocrinol, 31*(2), 134-156.
690 doi:10.1016/j.yfrne.2010.01.001
691 Noyes, P. D., Friedman, K. P., Browne, P., Haselman, J. T., Gilbert, M. E., Hornung, M. W., . . . Degitz, S. J.
692 (2019). Evaluating Chemicals for Thyroid Disruption: Opportunities and Challenges with in Vitro
693 Testing and Adverse Outcome Pathway Approaches. *Environ Health Perspect, 127*(9), 95001.
694 doi:10.1289/EHP5297
695 Olker, J. H., Korte, J. J., Denny, J. S., Hartig, P. C., Cardon, M. C., Knutsen, C. N., . . . Hornung, M. W.
696 (2019). Screening the ToxCast Phase 1, Phase 2, and e1k Chemical Libraries for Inhibitors of
697 Iodothyronine Deiodinases. *Toxicol Sci, 168*(2), 430-442. doi:10.1093/toxsci/kfy302
698 Olker, J. H., Korte, J. J., Denny, J. S., Haselman, J. T., Hartig, P. C., Cardon, M. C., . . . Degitz, S. J. (2021). In
699 vitro screening for chemical inhibition of the iodide recycling enzyme, iodotyrosine deiodinase.
700 *Toxicol In Vitro, 71*, 105073. doi:10.1016/j.tiv.2020.105073
701 Ouyang, X., Froment, J., Leonards, P. E. G., Christensen, G., Tollefsen, K. E., de Boer, J., . . . Lamoree, M.
702 H. (2017). Miniaturization of a transthyretin binding assay using a fluorescent probe for high
703 throughput screening of thyroid hormone disruption in environmental samples. *Chemosphere,*
704 *171*, 722-728. doi:10.1016/j.chemosphere.2016.12.119
705 Panda, S., & Kar, A. (2007). Apigenin (4',5,7-trihydroxyflavone) regulates hyperglycaemia, thyroid
706 dysfunction and lipid peroxidation in alloxan-induced diabetic mice. *J Pharm Pharmacol, 59*(11),
707 1543-1548. doi:10.1211/jpp.59.11.0012
708 Paul-Friedman, K., Martin, M., Crofton, K. M., Hsu, C. W., Sakamuru, S., Zhao, J., . . . Houck, K. A. (2019).
709 Limited Chemical Structural Diversity Found to Modulate Thyroid Hormone Receptor in the
710 Tox21 Chemical Library. *Environ Health Perspect, 127*(9), 97009. doi:10.1289/EHP5314
711 Paul Friedman, K., Papineni, S., Marty, M. S., Yi, K. D., Goetz, A. K., Rasoulpour, R. J., . . . Peffer, R. C.
712 (2016). A predictive data-driven framework for endocrine prioritization: a triazole fungicide case
713 study. *Crit Rev Toxicol, 46*(9), 785-833. doi:10.1080/10408444.2016.1193722
714 Paul, K. B., Hedge, J. M., Rotroff, D. M., Hornung, M. W., Crofton, K. M., & Simmons, S. O. (2014).
715 Development of a thyroperoxidase inhibition assay for high-throughput screening. *Chem Res*
716 *Toxicol, 27*(3), 387-399. doi:10.1021/tx400310w
717 Prapunpoj, P., & Leelawatwattana, L. (2009). Evolutionary changes to transthyretin: structure-function
718 relationships. *FEBS J, 276*(19), 5330-5341. doi:10.1111/j.1742-4658.2009.07243.x

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

719 Rabah, S. A., Gowan, I. L., Pagnin, M., Osman, N., & Richardson, S. J. (2019). Thyroid Hormone
720 Distributor Proteins During Development in Vertebrates. *Front Endocrinol (Lausanne), 10*, 506.
721 doi:10.3389/fendo.2019.00506
722 Radovic, B., Mentrup, B., & Kohrle, J. (2006). Genistein and other soya isoflavones are potent ligands for
723 transthyretin in serum and cerebrospinal fluid. *Br J Nutr, 95*(6), 1171-1176.
724 doi:10.1079/bjn20061779
725 Ren, X. M., & Guo, L. H. (2012). Assessment of the binding of hydroxylated polybrominated diphenyl
726 ethers to thyroid hormone transport proteins using a site-specific fluorescence probe. *Environ*
727 *Sci Technol, 46*(8), 4633-4640. doi:10.1021/es2046074
728 Renko, K., Hoefig, C. S., Dupuy, C., Harder, L., Schwiebert, C., Kohrle, J., & Schomburg, L. (2016). A
729 Nonradioactive DEHAL Assay for Testing Substrates, Inhibitors, and Monitoring Endogenous
730 Activity. *Endocrinology, 157*(12), 4516-4525. doi:10.1210/en.2016-1549
731 Renko, K., Hoefig, C. S., Hiller, F., Schomburg, L., & Kohrle, J. (2012). Identification of iopanoic acid as
732 substrate of type 1 deiodinase by a novel nonradioactive iodide-release assay. *Endocrinology,*
733 *153*(5), 2506-2513. doi:10.1210/en.2011-1863
734 Renko, K., Schache, S., Hoefig, C. S., Welsink, T., Schwiebert, C., Braun, D., . . . Schomburg, L. (2015). An
735 Improved Nonradioactive Screening Method Identifies Genistein and Xanthohumol as Potent
736 Inhibitors of Iodothyronine Deiodinases. *Thyroid, 25*(8), 962-968. doi:10.1089/thy.2015.0058
737 Richard, A. M., Judson, R. S., Houck, K. A., Grulke, C. M., Volarath, P., Thillainadarajah, I., . . . Thomas, R.
738 S. (2016). ToxCast Chemical Landscape: Paving the Road to 21st Century Toxicology. *Chem Res*
739 *Toxicol, 29*(8), 1225-1251. doi:10.1021/acs.chemrestox.6b00135
740 Richardson, S. J. (2002). The evolution of transthyretin synthesis in vertebrate liver, in primitive
741 eukaryotes and in bacteria. *Clin Chem Lab Med, 40*(12), 1191-1199. doi:10.1515/CCLM.2002.209
742 Richardson, S. J. (2007). Cell and molecular biology of transthyretin and thyroid hormones. *International*
743 *Review of Cytology - a Survey of Cell Biology, Vol 258, 258*, 137-193. doi:10.1016/S0074-
744 7696(07)58003-4
745 Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., . . . Makarov,
746 S. (2008). Homogeneous reporter system enables quantitative functional assessment of multiple
747 transcription factors. *Nat Methods, 5*(3), 253-260. doi:10.1038/nmeth.1186
748 Schreiber, G. (2002). The evolutionary and integrative roles of transthyretin in thyroid hormone
749 homeostasis. *J Endocrinol, 175*(1), 61-73. doi:10.1677/joe.0.1750061
750 Sushko, I., Novotarskyi, S., Korner, R., Pandey, A. K., Rupp, M., Teetz, W., . . . Tetko, I. V. (2011). Online
751 chemical modeling environment (OCHEM): web platform for data storage, model development
752 and publishing of chemical information. *J Comput Aided Mol Des, 25*(6), 533-554.
753 doi:10.1007/s10822-011-9440-2
754 Tan, S. W., & Zoeller, R. T. (2007). Integrating basic research on thyroid hormone action into screening
755 and testing programs for thyroid disruptors. *Crit Rev Toxicol, 37*(1-2), 5-10.
756 doi:10.1080/10408440601123396
757 Team, R. C. (2022). R: A language and environment for statistical computing. Vienna, Austria: R
758 Foundation for Statistical Computing. Retrieved from https://www.R-project.org/
759 Tetko, I. V. (2024). Tox24 Challenge. *Chemical Research in Toxicology*.
760 doi:10.1021/acs.chemrestox.4c00192
761 Tijani, J., Fatoaba, O., OO, B., & Petrick, L. (2016). Pharmaceuticals, endocrine disruptors, personal care
762 products, nanomaterials and perfluorinated pollutants: a review. *Environ Chem Lett, 14*, 27-49.
763 doi:10.1007/s10311-015-0537-z
764 Titus, S., Neumann, S., Zheng, W., Southall, N., Michael, S., Klumpp, C., . . . Austin, C. P. (2008).
765 Quantitative high-throughput screening using a live-cell cAMP assay identifies small-molecule
766 agonists of the TSH receptor. *J Biomol Screen, 13*(2), 120-127. doi:10.1177/1087057107313786

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0

767 Tomar, D., Khan, T., Singh, R. R., Mishra, S., Gupta, S., Surolia, A., & Salunke, D. M. (2012).
768 Crystallographic study of novel transthyretin ligands exhibiting negative-cooperativity between
769 two thyroxine binding sites. *PLoS One, 7*(9), e43522. doi:10.1371/journal.pone.0043522
770 USEPA. (2021). *ToxCast Database (invitroDB)*.
771 USEPA. (2022). *Availability of New Approach Methodologies (NAMs) in the Endocrine Disruptor
772 Screening Program (EDSP)*. (EPA-HQ-OPP-2021-0756-0002).
773 Van den Berg, K. J., van Raaij, J. A. G. M., Bragt, P. C., & Notten, W. R. F. (1991). Interactions of
774 halogenated industrial chemicals with transthyretin and effects on thyroid hormone levels in
775 vivo. *Archives of Toxicology*(65), 15-19.
776 van der Zalm, A. J., Barroso, J., Browne, P., Casey, W., Gordon, J., Henry, T. R., . . . Clippinger, A. J. (2022).
777 A framework for establishing scientific confidence in new approach methodologies. *Arch Toxicol,
778 96*(11), 2865-2879. doi:10.1007/s00204-022-03365-4
779 van Dijk, J., Gustavsson, M., Dekker, S. C., & van Wezel, A. P. (2021). Towards 'one substance - one
780 assessment': An analysis of EU chemical registration and aquatic risk assessment frameworks. *J
781 Environ Manage, 280*, 111692. doi:10.1016/j.jenvman.2020.111692
782 Vorberg, S., & Tetko, I. V. (2014). Modeling the Biodegradability of Chemical Compounds Using the
783 Online CHEmical Modeling Environment (OCHEM). *Mol Inform, 33*(1), 73-85.
784 doi:10.1002/minf.201300030
785 Wang, H., Tang, Y., & Lei, M. (2007). Models for binding cooperativities of inhibitors with transthyretin.
786 *Arch Biochem Biophys, 466*(1), 85-97. doi:10.1016/j.abb.2007.07.010
787 Wang, J., Hallinger, D. R., Murr, A. S., Buckalew, A. R., Simmons, S. O., Laws, S. C., & Stoker, T. E. (2018).
788 High-Throughput Screening and Quantitative Chemical Ranking for Sodium-Iodide Symporter
789 Inhibitors in ToxCast Phase I Chemical Library. *Environ Sci Technol, 52*(9), 5417-5426.
790 doi:10.1021/acs.est.7b06145
791 Yamauchi, K., Ishihara, A., Fukazawa, H., & Terao, Y. (2003). Competitive interactions of chlorinated
792 phenol compounds with 3,3′,5-triiodothyronine binding to transthyretin: detection of possible
793 thyroid-disrupting chemicals in environmental waste water. *Toxicology and Applied
794 Pharmacology, 187*(2), 110-117. doi:10.1016/s0041-008x(02)00045-5
795 Zhang, J. H., Chung, T. D., & Oldenburg, K. R. (1999). A Simple Statistical Parameter for Use in Evaluation
796 and Validation of High Throughput Screening Assays. *J Biomol Screen, 4*(2), 67-73.
797 doi:10.1177/108705719900400206
798 Zoeller, R. T., Tan, S. W., & Tyl, R. W. (2007). General background on the hypothalamic-pituitary-thyroid
799 (HPT) axis. *Crit Rev Toxicol, 37*(1-2), 11-53. doi:10.1080/10408440601123446
800
801

https://doi.org/10.26434/chemrxiv-2024-pwgq6-v2 **ORCID:** https://orcid.org/0000-0002-0007-2421 Content not peer-reviewed by ChemRxiv. **License:** CC BY-NC 4.0