Identification of hydrocarbon sulfonates as previously overlooked transthyretin ligands in the environment

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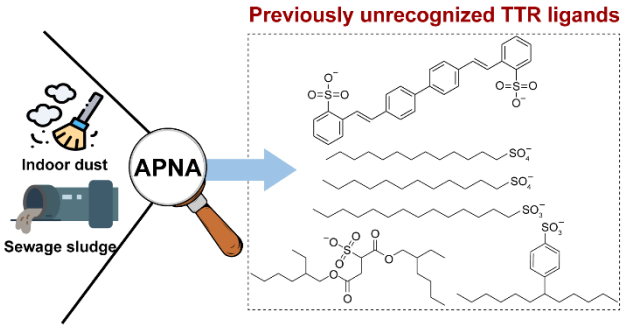

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Abstract

Incidences of thyroid disease, which has long been hypothesized to be partially caused by exposure to thyroid hormone disrupting chemicals (TDCs), have rapidly increased in recent years. However, only ~1% of the binding activity of human transthyretin (hTTR), an important thyroid hormone transporter protein, can be explained by known TDCs. In this study, we aimed to identify the major hTTR ligands in Canadian indoor dust and sewage sludge by employing protein-guided nontargeted analysis. hTTR binding activities were detected in all 11 indoor dust and 9 out of 10 sewage sludge samples (median 458 and 1134 μg T_4/g in dust and sludge, respectively) by the FITC-T_4 displacement assay. Through employing protein **A**ffinity **P**urification with **N**ontargeted **A**nalysis (APNA), 31 putative hTTR ligands were detected including perfluorooctane sulfonate (PFOS). Two of the most abundant ligands were identified as hydrocarbon surfactants (e.g., dodecyl benzenesulfonate), which were confirmed by authentic chemical standards. Structure-activity relationships (SAR) of hydrocarbon surfactants were explored by investigating the binding activity of 11 hydrocarbon surfactants to hTTR. Optimal carbon chain length (C_{12-14}) was found to achieve a high binding affinity. By employing *de novo* nontargeted analysis, another abundant ligand was surprisingly identified as a di-sulfonate fluorescent brightener, 4,4'-Bis(2-sulfostyryl)biphenyl sodium (CBS). CBS was validated as a nM-affinity hTTR ligand with an IC_{50} of 345 nM. In total, hydrocarbon surfactants and fluorescent brightener could explain 1.92-17.0% and 5.74-54.3% of hTTR binding activities in dust and sludge samples, respectively, whereas PFOS only contributed <0.0001% to the activity. Our study revealed for the first time that hydrocarbon sulfonates are previously overlooked hTTR ligands in the environment.

Keywords: TTR; Hydrocarbon surfactants; CBS; Nontargeted analyses; Bioanalytical equivalent concentration

Synopsis: Hydrocarbon surfactants and fluorescent brightener are previously overlooked TTR ligands in the environment.

Introduction

Thyroid hormones (THs) play a pivotal role in regulating many physiological processes in organisms such as growth, development, and energy metabolism.[1] Disruption of THs levels can impact the development of the central nervous system, especially during fetal development,[2] and is associated with neurological disorders including Alzheimer's disease (AD)[3]. Epidemiological studies have revealed an association between exposure to thyroid hormone disrupting chemicals (TDCs) and the increased risk of thyroid-related diseases.[4-6] Transthyretin (TTR) is a major thyroxine (T_4) transport protein in serum that has been demonstrated to mediate the toxicity of many TDCs. TDCs can compete with endogenous T_4 for binding to TTR, which can lead to the increased clearance of free T_4 and the disruption of THs homeostasis.[7, 8] In addition to THs disruption, binding to TTR can also mediate the transport of chemical pollutants across placental and blood-brain barriers into various compartments (*e.g.,* fetus and brain), which may induce a multitude of adverse effects.[9-11] Thus, the identification of environmental chemicals binding to TTR is important for understanding their potential health risks.

TTR is a tetramer protein with a large binding pocket in the middle channel. Multiple compound classes have been reported to bind to TTR, including hydroxylated polybrominated diphenyl ethers (OH-PBDEs)[12], phenolic disinfection products (phenolic-DBPs)[13], hydroxylated and sulfated metabolites of polychlorinated biphenyls (PCBs)[14], per- and polyfluoroalkyl substances (PFASs)[15], and many others.[16] Despite the extensive studies on TTR, known environmental TTR ligands (*e.g.,* PFASs) can only explain 1.2% of the total TTR activity of house dust[17], while the vast majority of TTR ligands in the environment remain unidentified. This knowledge gap should largely result from the vast number of synthetic chemicals manufactured worldwide (>350,000 until 2019)[18] and the limited capacity of conventional toxicity testing methods. Effect-directed analysis (EDA) is a promising tool for the identification of unknown toxic compounds in complex environmental matrices.[19] However, EDA is time-consuming and, more importantly, is prone to high false discovery rates due to the co-elution of chemicals in the same fractions. For example, although strong TTR

binding activity was detected for a standard dust sample (SRM 2585), TTR ligands identified by EDA (e.g., synthetic musks, PFASs, and organophosphates) could barely explain the total effects.[20] Similarly, triclosan and nonylphenol were demonstrated to contribute to the TTR binding activity of a sediment sample by EDA, but only <1% of the activity could be explained.[21] Together, these results underscore the need to systematically identify unknown TTR ligands in the environment.

Individual testing of each of the 350,000 compounds for TTR activity is infeasible due to the significant cost requirement and the unavailability of standards. To address this challenge, we have developed a "top-down" approach termed "protein **A**ffinity **P**urification with **N**ontargeted **A**nalysis (APNA)"[22, 23] for the identification of ligands at the exposome-wide level. The APNA method is fundamentally different from conventional EDA methods in that it uses protein affinity to directly isolate bioactive chemicals from environmental mixtures in an unbiased manner. To date, APNA has been successfully applied to identify novel ligands binding to human nuclear receptors,[24, 25] transport proteins,[26, 27] and even bacterial enzymes[28, 29]. Inspired by those successful applications, we herein employed the APNA method to systematically identify human TTR (hTTR) ligands in indoor dust and sewage sludge samples. Surprisingly, hydrocarbon sulfonates including surfactants and a fluorescent brightener (*i.e.*, 4,4'-Bis(2-sulfostyryl)biphenyl sodium (CBS)), were identified as major hTTR ligands in the environment, highlighting the need to re-evaluate their chemical safety in the future.

Materials and Methods

Chemicals and Reagents. Authentic standards including several hydrocarbon surfactants, perfluorooctanesulfonic acid (PFOS), thyroxine (T_4), rosiglitazone, and fluorescein isothiocyanate (FITC) (isomer I) were purchased from Sigma-Aldrich (Oakville, ON, CA). Docusate sodium and sodium tridecyl sulfate were obtained from Chem Service (West Chester, PA, USA). CBS was obtained from Alfa Chemistry (Ronkonkoma, NY, USA). Detailed information regarding the chemical standards is provided in Table S1 of the Supporting Information (SI). Native hTTR protein purified

from human plasma (purity ≥ 95%) was obtained from Sigma-Aldrich (Catalog#: 529577; Oakville, ON, CA). LC-MS grade acetonitrile, methanol, water, and ammonium acetate were purchased from Fisher Scientific (Ottawa, ON, CA).

Environmental sample collection and extraction. A total of 11 indoor dust samples and 10 sewage sludge samples were collected and extracted as previously described.[26] Detailed information on the sample preparation is provided in the SI.

FITC-T_4 Displacement Assay. The binding affinities of pulled-out chemicals or extracts of environmental samples to hTTR were determined individually by a fluorescein−thyroxine (FITC-T_4) displacement assay. Synthesis of the fluorescence probe FITC-T_4 and development of the displacement assay were completed according to previous studies with minor modifications,[30, 31] and a detailed description of the method is provided in the SI. For extracts of indoor dust and sewage sludge, the uppermost concentration was capped at 0.5 g/L due to the significant signal interference caused by the intense coloration of the extracts at exposure concentrations exceeding 0.5 g/L. It should be noted that the native form of hTTR protein, purified directly from human plasma rather than the recombinant His-tagged hTTR from *E. coli*, was used for the assay to cross-validate the APNA results in order to avoid the impact of the His tag on potential binding activities.

Overexpression of Recombinant His-hTTR Protein. In this study, the His-tagged hTTR protein was expressed in *E. coli* BL21 (DE3) cells (Novagen, WI, USA). The expression vector of human His-tagged TTR protein was obtained from GenScript corporation (Piscataway, NJ, USA). More details are provided in the SI.

Protein Affinity Pulldown. In this study, the APNA method[22, 24-26] was employed to identify environmental pollutants binding to hTTR in indoor dust and sewage sludge. In brief, crude lysates (250 µL) of *E. coli* cells overexpressing His-tagged hTTR protein were incubated with 3 µL of sample extract (*i.e.*, indoor dust or sewage sludge) in a 96-well plate. 5 µL of His-select nickel magnetic agarose beads (H9914, Sigma-Aldrich) were then added and the whole plate was incubated at 4 °C for 30 min in a rotator at 20 rpm to allow the formation of protein-ligand complexes. The experiments were performed in triplicate ($N = 3$). Lysates of wild-type *E. coli* cells were used as negative

controls. After incubation, the 96-well plate was placed on a magnetic field plate to separate the beads. The supernatant was removed, and the magnetic beads were washed 3 times using 100 µL of wash buffer (50 mM Tris, 300 mM sodium chloride, and 30 mM imidazole, pH 8.0). Then, 100 µL of elution buffer (50 mM Tris, 300 mM sodium chloride, and 300 mM imidazole, pH 8.0) was added to wash-off the protein-ligand complex from the magnetic beads. The eluted solution was then transferred to a Zeba™ spin 7k MWCO desalting plate (Thermo Fisher Scientific). Following centrifugation at 1000 *g* for 2 minutes, the eluted solution was collected and transferred to a 1.5 mL Eppendorf tube. The sample was then dried down by a speed vacuum at room temperature and 100 µL of cold methanol was added to denature the His-tagged hTTR protein. The sample was vortexed for 1 minute, and centrifuged again for 30 minutes at 14,000 *g*. The supernatant was finally transferred to sample vials for LC-MS/MS analysis.

Ligand Identification by Nontargeted Analyses. LC-MS/MS analysis was conducted by use of a Q Exactive mass spectrometer coupled online with a Vanquish ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific, Waltham, MA, USA). Complete details of the instrument methods are provided in the SI. Nontargeted analyses were accomplished with an in-house R program as described in our previous studies.[32] A putative lock mass algorithm (PLMA) was applied for post-acquisition calibration of mass spectra before peak picking.[32] The 'XCMS' R package[33] was used for peak detection with a mass tolerance of 2.5 ppm. The peak features were aligned across samples with a mass tolerance of 2.5 ppm and retention time window of 20 seconds after retention time adjustment. In this study, to ensure specificity, lysates of wild-type *E. coli* cells were used as the negative control. The ratio between the peak abundance from *E. coli* overexpressing His-tagged hTTR to that from wild-type *E. coli* was calculated for each peak feature. The *P* value of the difference between the two groups was also determined by student's t-test. Only the features exhibiting greater peak intensities (fold change > 5, p value < 0.05) in the overexpressed hTTR group compared to the wild-type *E. coli* group were considered as potential ligands. *E. coli* overexpressing His-tagged hTTR without incubation with

dust/sludge extracts was also employed as a second negative control. The pulled-out LC-MS features were further filtered by using the second negative control with the same cutoffs. Isotopic peaks and adducts were excluded by matching chromatographic peaks and theoretical mass differences. The final differentiated peak list from the output of the R program was manually checked by use of Qual Browser in Xcalibur software. Then, the differentiated peaks were searched against the United States Environmental Protection Agency (U.S. EPA) Toxic Substances Control Act Chemical Substance Inventory (TSCA Inventory)[34] and the Network of Reference Laboratories, Research Centers, and Related Organizations for Monitoring of Emerging Environmental Substances (NORMAN) Suspect List Exchange database[35] using an in-house R program.[32] A mass tolerance of 2.5 ppm was used. Confidence levels were assigned to all identities according to the Schymanski scale.[36]

Absolute quantification. The concentrations of identified hTTR ligands were determined by LC-MS/MS in this study. External calibration curves were constructed for each chemical by using corresponding authentic standards. Please see the SI for more details.

Molecular docking. AutoDock Vina 1.1.2[37] was used to predict the binding modes of identified chemicals to hTTR (PDB code: 2ROX). The detailed procedures and parameters are available in the SI.

Calculation of BEQ. In this study, the contribution of the identified hTTR ligands to the hTTR binding activities of environmental samples was estimated by comparing the bioanalytical equivalent concentration (BEQ) from bioanalysis (BEQ_{bio}) and the BEQ from chemical analysis (BEQ_{chem}).[4] The BEQ_{bio} of environmental samples toward hTTR binding were estimated on the basis of IC_{50} values determined by the FITC-T_4 displacement assay, with T_4 as a reference compound, using eq 1.

$$BEQ_{bio} = \frac{IC_{50(T_4)}}{IC_{50(\text{environmental samples})}} \quad (1)$$

To calculate the BEQ_{chem}, the relative potency (REP_i) of each tested chemical (i) relative to T_4 was estimated on the basis of its IC_{50} using eq 2.

$$REP_i = \frac{IC_{50(T_4)}}{IC_{50(i)}} \quad (2)$$

Then, the BEQ_{chem} was estimated by multiplying the quantified concentrations of each chemical (C_i) in the environmental samples by their respective REP values (eq 3).

$$BEQ_{chem} = \sum_{i=1}^{n} REP_i \times C_i \quad (3)$$

Finally, the contributions to the observed hTTR binding activities were determined by eq 4:

$$\text{Contribution } (\%) = \frac{BEQ_{chem}}{BEQ_{bio}} \times 100\% \quad (4)$$

Please refer to SI for more details about statistics.

Results and Discussion

Widespread hTTR binding activities in indoor dust and sewage sludge. To investigate the potential occurrence of hTTR ligands in the environment, we employed the FITC-T_4 probe-based fluorescence displacement assay developed in previous studies.[30, 38] The FITC-T_4 probe shows a fluorescence enhancement at 518 nm when binding to the hTTR protein, probably due to the impact on the intersystem crossing effect of the iodine atom. If a competitive hTTR ligand is present, the probe would be displaced from hTTR, resulting in a decrease in the fluorescence signal.[30] The FITC-T_4 probe was synthesized using a one-step amine coupling reaction followed by a GPC column purification. The identity and purity (>99%) of the probe was confirmed by high resolution mass spectrometry (Figure S1a and S1b). To test its function, the synthesized probe was incubated with native hTTR protein for fluorescence measurement. The fluorescence intensity at 518 nm increased with increasing concentrations of FITC-T_4 and reached saturation at about 1 μM of FITC-T_4 when incubating with a fixed concentration (1 μM) of hTTR (Figure S1c). To further benchmark the assay for competitive ligand screening, three well-known hTTR ligands (*i.e.*, thyroid hormone T_4, 6-OH-BDE-47, and PFOS) were individually incubated with the FITC-T_4 probe (130 nM) and hTTR protein (90 nM). As expected, all three

chemicals displaced the FITC-T_4 probe from hTTR protein as evidenced by the decrease in the fluorescence signal in a dose-dependent manner (Figure S2). Moreover, the REP values of 6-OH-BDE-47 (REP = 1.83) and PFOS (REP = 0.49) relative to T_4 measured in our study are comparable to those reported in previous studies (REP of 2 and 0.4 for 6-OH-BDE-47[39] and PFOS,[17] respectively), demonstrating the validity of the synthesized FITC-T_4 probe.

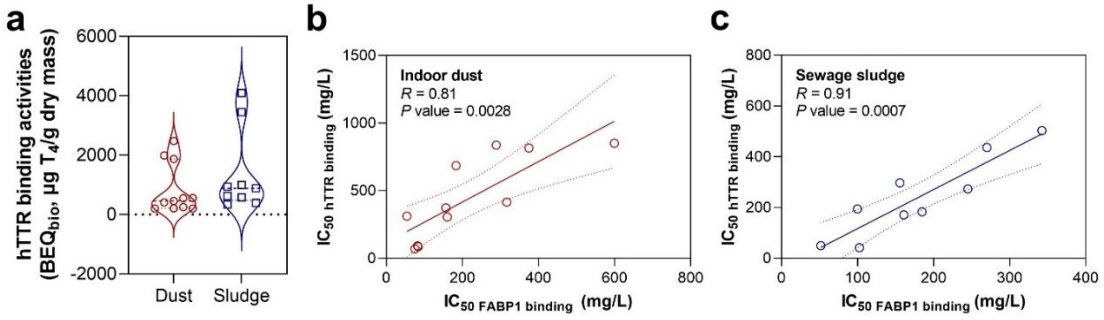

Figure 1. Widespread hTTR binding activity in the environment. (**a**) Detected hTTR binding activities of indoor dust and sewage sludge extracts expressed as BEQ_{bio} (μg T_4/g dry mass). (**b**) and (**c**), correlation analysis of the binding activities between hTTR and FABP1 across indoor dust and sewage sludge extracts, respectively. The hTTR binding activity was determined by a FITC-T_4 fluorescence displacement assay. $N = 3$. All data were normalized to solvent control. The FABP1 binding potencies were adopted from a previous study.[26]

We then moved forward to use the FITC-T_4 displacement method for the measurement of hTTR activity in environmental extracts. A total of 11 indoor dust samples and 10 sewage sludge samples were extracted and tested for their hTTR binding potencies. Except for one sludge sample (*i.e.*, sludge sample S5), all the extracted indoor dust and sewage sludge samples showed marked hTTR binding activities, as depicted by the full dose-response curves in Figure S3 and S4. The T_4-BEQ_{bio} concentrations of the indoor dust extracts were estimated to be 201 to 2477 μg/g dust (median: 458 μg/g dust), while those of sewage sludge extracts were higher (range: 340 to 4089 μg/g sludge; median: 881 μg/g sludge) (Figure 1a and Table S2). The strong hTTR binding activity of indoor dust samples was not surprising as extensive hTTR binding activity has been previously reported for dust samples collected from Japan and

the United States.[17, 40, 41] Wastewater-based monitoring has long been used as a promising tool to measure the collective consumption or chemical exposure of humans.[42] The sewage sludge samples were collected from two biggest wastewater treatment plants in Toronto, and thus the strong hTTR binding activity of the sewage sludge extracts suggested the potential population-wide exposure of TDCs to the Toronto population.

In our recent study, the binding activities of human liver fatty acid binding protein 1 (FABP1) and peroxisome proliferator-activated nuclear receptor γ (PPARγ) ligand binding domain (LBD) which are important target proteins of environmental obesogens were also determined for the same dust and sludge samples.[26] This provided an opportunity to compare the binding activities of three proteins (*i.e.,* hTTR, FABP1, and PPARγ) across the samples. A significantly positive correlation was observed between the hTTR binding activities and the FABP1 binding activities across indoor dust (R = 0.81, P = 0.0028, Figure 1b) and sewage sludge (R = 0.91, P = 0.0007, Figure 1c) samples. Positive correlation was also observed between hTTR and PPARγ LBD but the correlation was weaker (Figure S5). The results demonstrated that these three proteins might share common ligands in the environment, particularly between hTTR and FABP1. This is very interesting, as hTTR and FABP1 are both major transport proteins, and they have been reported to share some common endogenous ligands (*e.g.,* arachidonate).[43, 44]

Nontargeted identification of hTTR ligands by APNA. To identify the primary hTTR ligands in the environmental samples, the APNA approach was employed. The His-tagged hTTR was overexpressed in *E. coli* and its amino acid sequence was verified by LC-MS/MS (Figure S6). For proof of concept, the APNA method was first benchmarked by incubating a mixture of T_4 and 6-OH-BDE-47 with recombinant His-tagged hTTR protein. As shown in Figure S7a, these two well-known hTTR ligands, with binding affinities at nanomolar range (*i.e.,* T_4 and 6-OH-BDE-47[30]), were significantly pulled-out by His-tagged hTTR but not by the wild-type *E. coli* lysates. Meanwhile, in another independent validation experiment, the mixture of T_4 and rosiglitazone were tested against three different protein targets, including His-tagged

hTTR, His-tagged FABP1, and His-tagged PPARγ LBD, separately. As expected, T_4 and rosiglitazone were only pulled-out by their corresponding target proteins (i.e., T_4 for hTTR; rosiglitazone for FABP1 and PPARγ LBD), demonstrating the high selectivity of the APNA approach (Figure S7b). These results together demonstrated that the APNA approach can be used to isolate hTTR ligands with good selectivity.

Subsequently, we employed the APNA method to directly identify environmental hTTR ligands from the indoor dust and sewage sludge extracts containing thousands of co-occurring chemicals. Among the 19,175 and 19,028 LC-MS features detected in pooled indoor dust and sewage sludge extracts, respectively, 17 and 14 features were specifically pulled-out by the *E. coli* lysates overexpressing His-tagged hTTR with >5-fold higher abundances than the wild-type *E. coli* lysates (negative control) ($P < 0.05$; Figure 2a and 2b). The peak shapes, *m/z*, and retention times (RTs) of all the pulled-out features were confirmed by manual inspection. During the data inspection stage, one additional feature (*i.e.,* *m/z* = 325.1841, RT = 5.86 min), which was found to be significantly pulled-out by His-tagged hTTR protein ($P < 0.005$) but with a lower fold change (fold change = 3.82) due to its high background in the LC-MS instrument, was manually added to the pulled-out list. After removing the repetitive features, a total of 31 nonredundant LC-MS features were detected as putative hTTR ligands across the dust and sludge extracts (Table S3).

Through suspect screening against the TSCA Inventory database and the NORMAN database, the structures of 3 LC-MS features were tentatively assigned, including PFOS (*m/z* = 498.9302, RT = 5.02 min, $[C_8F_{17}O_3S]^-$, mass error = -0.035 ppm) (Figure 2c). The detection of PFOS as a hTTR ligand was not surprising since its hTTR binding activity has been widely verified in previous studies by several different approaches such as TTR binding assays with FITC-T_4[45] or ^{125}I-labeled T_4[15] and *in silico* modeling[46]. These results verified our APNA method for the identification of hTTR ligands from environmental mixtures. However, some well-known hTTR ligands, such as OH-PBDEs,[39, 47] were not detected by the APNA method which was likely due to their extremely low concentrations in environmental samples (typically at low ng/g levels[48]). Indeed, even PFOS exhibited a peak intensity that was several orders of magnitude

lower than that of other putative ligands. This indicated that previously known hTTR ligands might contribute only minorly to the hTTR activity, which was consistent with a previous study which demonstrated that known chemicals only explained 1.2% of hTTR activity in indoor dust.[17]

We then moved forward to assign the structures of other previously unknown hTTR ligands. By searching against the TSCA database, the ligands *m/z* = 325.1841 and *m/z* = 353.2154 were assigned as dodecyl benzenesulfonate and tetradecyl benzenesulfonate, respectively. Their identities were supported by the detection of a characteristic MS^2 fragment of *m/z* = 183.0113 ($[C_8H_7O_3S]^-$, mass error = -0.738 ppm) corresponding to the ethylene-substituted benzenesulfonate.[49, 50] Moreover, the fragment ion *m/z* = 79.9560 ($[SO_3]^-$, mass error = -1.363 ppm) further suggested they contained a sulfonate group. The identity of *m/z* = 325.1841 was confirmed as dodecyl benzenesulfonate by comparing its MS^2 spectrum with that of an authentic standard (Figure 2d). However, as shown in Figure 2e, the RT of the other putative ligand *m/z* = 353.2154 from the sample extracts (RT = 6.26 min) did not match to the standard of tetradecyl benzenesulfonate (RT = 6.45 min). After careful inspection, the MS^2 spectrum of the putative ligand was found to be similar to that of the standard, yet the relative intensities of fragments *m/z* = 183.0113 and *m/z* = 170.0038 were different (Figure 2f and g). This demonstrated that the putative ligand might be an isomer of tetradecyl benzenesulfonate, as alkylbenzene sulfonates are known to be manufactured as a mixture of many isomers. Indeed, we noted that the retention time (RT = 6.27 min) and MS^2 spectrum of the minor peak of the tetradecyl benzenesulfonate standard matched to the tentative ligand (Figures 2h). Collectively, we concluded that the putative ligand *m/z* = 353.2154 should be the internal/branched isomer of tetradecyl benzenesulfonate due to the fact that 1) branched isomers of compounds are known to elute earlier than their linear isomers (*i.e.*, the standard of tetradecyl benzenesulfonate) on reversed-phase columns,[51] and 2) the dominance of the MS^2 fragment *m/z* = 183.0113 from the branched isomer might be generated through the radical-induced cleavage of the branched side chain.

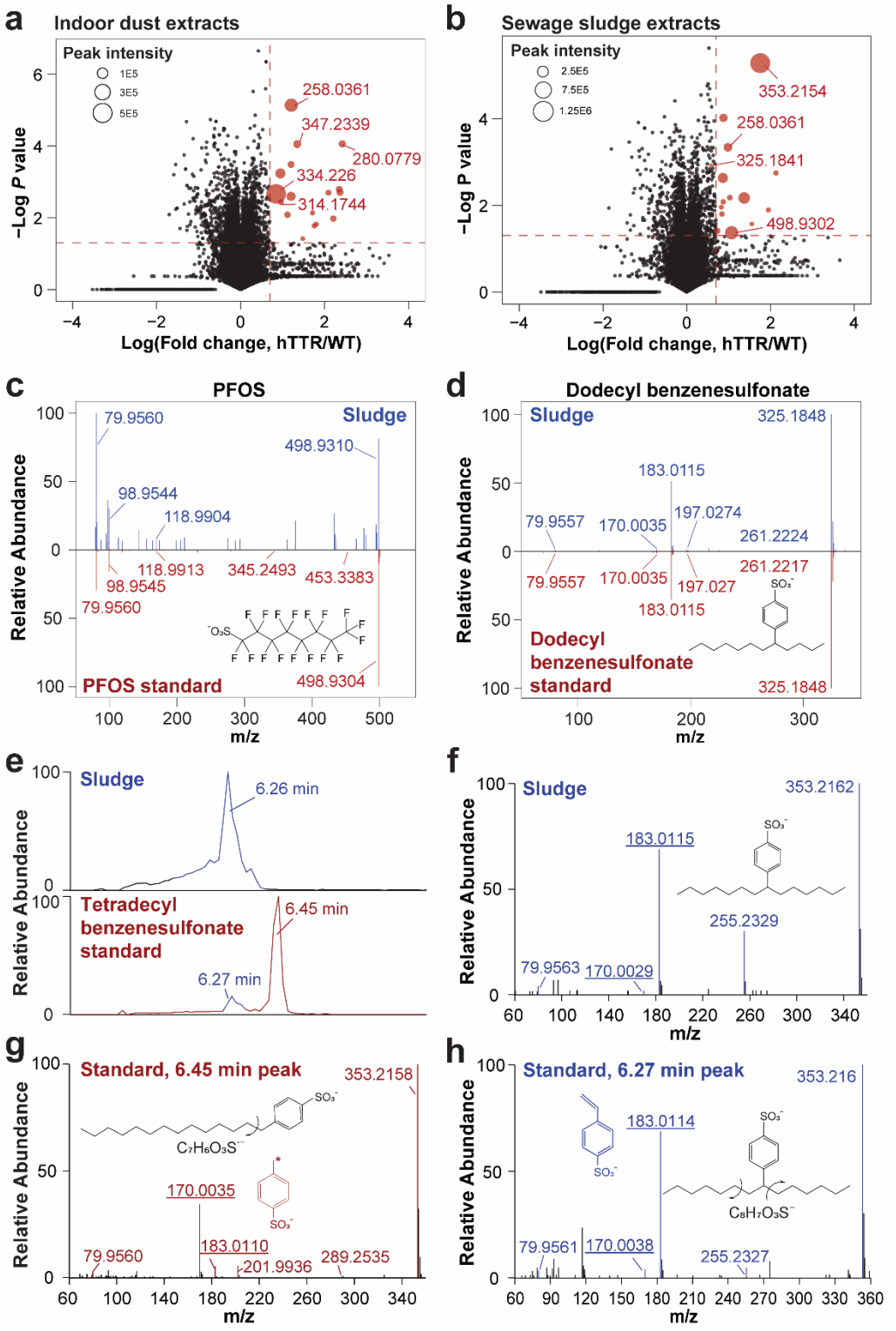

Figure 2. Identification of hTTR ligands by APNA. (a) and **(b)** Volcano plots representing the log-transformed fold changes and corresponding *P* values of each LC-MS feature detected in the pooled indoor dust and sewage sludge extracts. Red dots indicate LC-MS features having significantly greater abundances (fold change >5, $P < 0.05$) in *E. coli* lysates overexpressing His-tagged hTTR protein than the negative

control. Isotopic features and adducts were removed. Dot size (only for the upper right quadrant) represents the peak intensity for each pulled-out compound. (**c**) and (**d**) MS^2 spectra of PFOS and dodecyl benzenesulfonate, respectively, from sludge samples and matching to authentic standards. **(e)** Retention time of putative ligand *m/z* = 353.2154 matching to an authentic standard of tetradecyl benzenesulfonate. **(f)**, **(g)**, and **(h)** MS^2 spectra of putative ligand *m/z* = 353.2154, major peak in the standard (RT = 6.45 min), and minor peak in the standard (RT = 6.27 min). For the two alkylbenzene sulfonates, pictured above is one potential isomer for each compound, of which there are many possible isomers.

Overall, two major hTTR ligands were identified as hydrocarbon sulfonates. Identification of hydrocarbon sulfonates as hTTR ligands was unexpected but not completely surprising, as their structures are similar to PFOS. Hydrocarbon sulfonates are widely used as anionic surfactants in various household, industrial, and institutional applications. For instance, they appear in cleaner, lubricating agent, emulsifier, paint additives, and opacifer with an annual aggregated product volume of 100,000 to 500,000 pounds in the United States, for just dodecyl benzenesulfonate alone, according to Chemical Data Reporting (CDR).[52] Due to their high production volumes and wide applications, these compounds have been detected in indoor dust and sewage sludge samples at extremely high concentrations (mg/g levels), at ~6 orders of magnitude higher than those of PFOS in the same samples.[26]

Evaluation of hTTR binding activities of hydrocarbon surfactants. We further employed the FITC-T_4 displacement assay to cross-validate the binding activity of these newly identified hTTR ligands by using their commercially available standards. In line with the APNA results, dodecyl benzenesulfonate showed strong binding potency to hTTR with the IC_{50} and REP values of 9.57 μM and 0.0238, respectively (Figure 3a), whereas the hTTR binding activity of tetradecyl benzenesulfonate was much weaker in that only an IC_{10} value could be determined (IC_{10} = 2.74 μM; Figure 3b). It should be noted that the hTTR binding potency of tetradecyl benzenesulfonate was obtained by a linear/external isomer, not the branched/internal isomer (not commercially available) which was initially pulled-out from environmental samples by the hTTR protein. Previous studies have reported the stronger protein binding activities

of branched fatty acids than linear isomers.[53] This might lead to the underestimation of the hTTR binding potency of tetradecyl benzenesulfonate in the environment. Future studies are warranted to investigate isomer-specific binding of hydrocarbon surfactants to hTTR.

Moreover, as discussed above (Figure 1b and 1c), we found that the hTTR and FABP1 proteins may share common ligands in the tested indoor dust and sewage sludge samples. Previously, we demonstrated that several hydrocarbon surfactants including alkyl benzenesulfonate, alkyl sulfate, and alkyl sulfonate were predominant synthetic ligands of FABP1 in indoor dust and sewage sludge samples.[26] Motivated by this, we further included 11 hydrocarbon surfactants in the FITC-T_4 displacement assay to test their hTTR binding activities (Table S4). These chemicals usually have high background contaminations in the LC-MS instrument and thereby could be missed by our original screening algorithm. Among the 11 tested hydrocarbon surfactants, four of them including dodecyl sulfate (Figure 3c), tridecyl sulfate (Figure 3d), tetradecanesulfonate (Figure 3e), and docusate (Figure 3f) showed relatively strong binding activities towards the hTTR protein, with the IC_{50} and REP values ranging from 9.39 to 14.5 μM and 0.0152 to 0.0234, respectively. In contrast, hydrocarbon surfactants with a too short (e.g., C_2, C_7, and C_8) or too long (C_{16} and C_{18}) carbon chain length displayed weak or even no hTTR binding (IC_{50} > 50 μM and REP < 0.005) (Figure S8 and Figure S9). We conducted molecular docking with AutoDock Vina[37] to predict the binding mode of hydrocarbon surfactants to the hTTR protein, and included PFOS in the docking analysis for comparison. As shown in Figure S10a, PFOS could fit into the interior of the ligand binding pocket of hTTR with its sulfonic acid group protruding towards the surface and its fluorinated tail adopting an extended conformation (binding energy = -8.6 kcal/mol). Its sulfonic acid group formed a salt bridge with Lys15, which was consistent with previous observations[45] and demonstrated the accuracy of the molecular docking analysis. Then, by taking dodecyl benzenesulfonate (binding energy = -6.5 kcal/mol) as an example, we found that alkyl benzenesulfonate interacted with hTTR in a similar manner to PFOS, except that its hydrophobic tail bent inside the pocket interior (Figure S10b). Dodecyl benzenesulfonate also formed a salt bridge with

Lys15' and hydrophobic contacts with Ala108, Thr119, Leu17, Leu110, Thr106', Ala108', Leu17' and Leu110'. An additional anion-π interaction was also found to form between Lys15 and the benzene ring. Since hydrophobic contacts play a vital role in stabilizing the binding orientation, we deduced that smaller hydrocarbon surfactants (<C_8) could not form strong enough hydrophobic interactions with hTTR and would thus act as weaker hTTR ligands. In contrast, the alkyl chain of larger (>C_{16}) surfactants may have difficulty fitting into the hTTR binding pocket, resulting in low binding affinities. The molecular docking analysis provided a plausible explanation for the structure-activity relationships (SAR) of hydrocarbon surfactants.

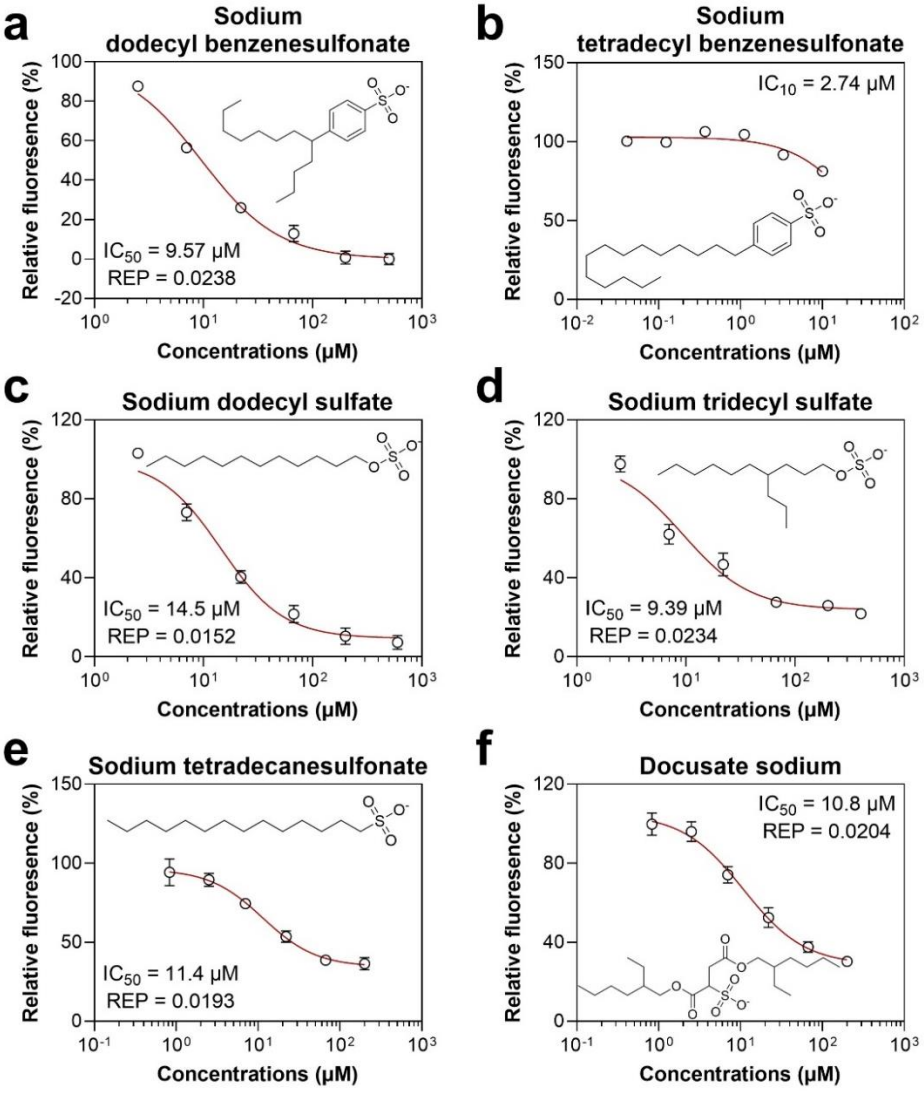

Figure 3. Binding of six hydrocarbon surfactants to native hTTR protein purified from human plasma. The binding activity was determined by a fluorescence

displacement assay. $N = 3$. All data were normalized to solvent control. REP: relative potency to T_4.

Identification of a fluorescent brightener as a nM affinity hTTR binder. Note that only 3 of the 31 nonredundant putative hTTR ligands were identified through database searching, probably because they were outside the TSCA or NORMAN chemical libraries, or they were ionized in a unique way (*e.g.,* multiple charged ions). We decided to employ *de novo* structural assignment for other putative hTTR ligands beyond the initial suspect screening. The LC-MS feature with a *m/z* of 258.0361 and retention time of 3.90 min attracted our attention since it was pulled-out both from indoor dust and sewage sludge extracts with a marked fold change > 10 and *P* value < 0.001. By carefully inspecting the isotopic distribution ($\Delta m/z$ = 0.5 Da), this feature was unexpectedly assigned as a doubly charged ion (Figure S11a). Its chemical formula was assigned as $[C_{28}H_{20}O_6S_2]^{2-}$ with a mass error of 1.88 ppm. The MS^2 fragment of *m/z* = 79.9560 (corresponding to $[SO_3]^-$) further suggested it was a sulfonate. The sequential neutral loss of two SO_2 (*i.e., m/z* = 226.0544 $[C_{28}H_{20}O_6S_2\text{-}SO_2]^{2-}$ and *m/z* = 194.0730 $[C_{28}H_{20}O_6S_2\text{-}2SO_2]^{2-}$) clearly demonstrated the presence of two sulfonate groups in the molecule. By re-searching against the NORMAN Suspect List Exchange database using the double charge[35], it was identified as CBS (CASRN: 27344-41-8). To confirm its identity, we purchased the authentic standard (purity > 99%). As illustrated in Figure 4a and 4b, the RT and the MS^2 spectrum of the feature pulled-out from the sample extracts matched exactly with the authentic standard of CBS, confirming the identity of *m/z* = 258.0361. In addition to CBS, we also detected four additional doubly charged chemicals in the indoor dust samples (Figure S11b to e). However, we were not able to assign their structures because they were outside the TSCA or NORMAN chemical databases. The MS information of these pulled-out chemicals has been uploaded to our "environmental Chemical-Protein Interaction Network (eCPIN)" database (https://penggroup.shinyapps.io/ecpin/)[22], which is freely accessible. It would be very interesting for colleagues working in nontargeted analysis to assign the structures of these unknown hTTR ligands, and check if these ligands are detected in

other samples of interest (*e.g.,* human cohort blood samples).

The structure of CBS is very unique compared to previously known hTTR ligands. This was interesting and demonstrated that APNA could identify novel ligands with completely new chemotypes. We moved forward to validate its bioactivity through the FITC-T_4 displacement assay as mentioned above. Supporting the APNA results, a dose-dependent reduction in fluorescence intensity was observed (Figure 4c) with an IC_{50} value of 0.345 μM, which confirmed the strong interaction between CBS and the hTTR protein. The REP value of CBS was determined to be 0.638, which was greater than PFOS (REP 0.49) and all the hydrocarbon surfactants tested in this study (Table S4). Through molecular docking, we found that the two sulfonate groups of the CBS molecule could form two hydrogen bonds with Thr123' and Ser117', and one salt bridge with Arg104' on each end of the hTTR ligand binding pocket (inner and entrance), which resulted in a stable binding orientation with a binding energy of -9.9 kcal/mol (Figure S10c). Moreover, hydrophobic contacts with Thr106', Ala108', Leu110', Ala108, Leu110, and Leu17 were also found. The special binding mode of CBS deriving from its unique doubly charged structure may provide an explanation for its strong hTTR binding potency.

Then, to better understand the potential environmental occurrence of CBS, we decided to quantify CBS in the selected dust and sludge samples. CBS was detected in all 11 indoor dust and 10 sewage sludge samples, at 0.17 to 3.55 μg/g (median: 1.69 μg/g) in indoor dust and 0.96 to 238 μg/g (median: 17.4 μg/g) in sewage sludge (Figure 4d and Table S5). The concentrations of CBS were about 3~4 orders of magnitudes higher than those of PFOS in the same samples, but ~10 times lower than those of hydrocarbon surfactants.[26] Two very recent studies from the Zeng group reported the occurrence of CBS in indoor dust and sludge collected from China.[54, 55] The detected concentrations of CBS in our study were comparable to the results from the Zeng et al studies (dust: 0.059 to 4.04 μg/g; sludge: 0.013 to 8.35 μg/g). These results demonstrated its ubiquitous presence in the environment.

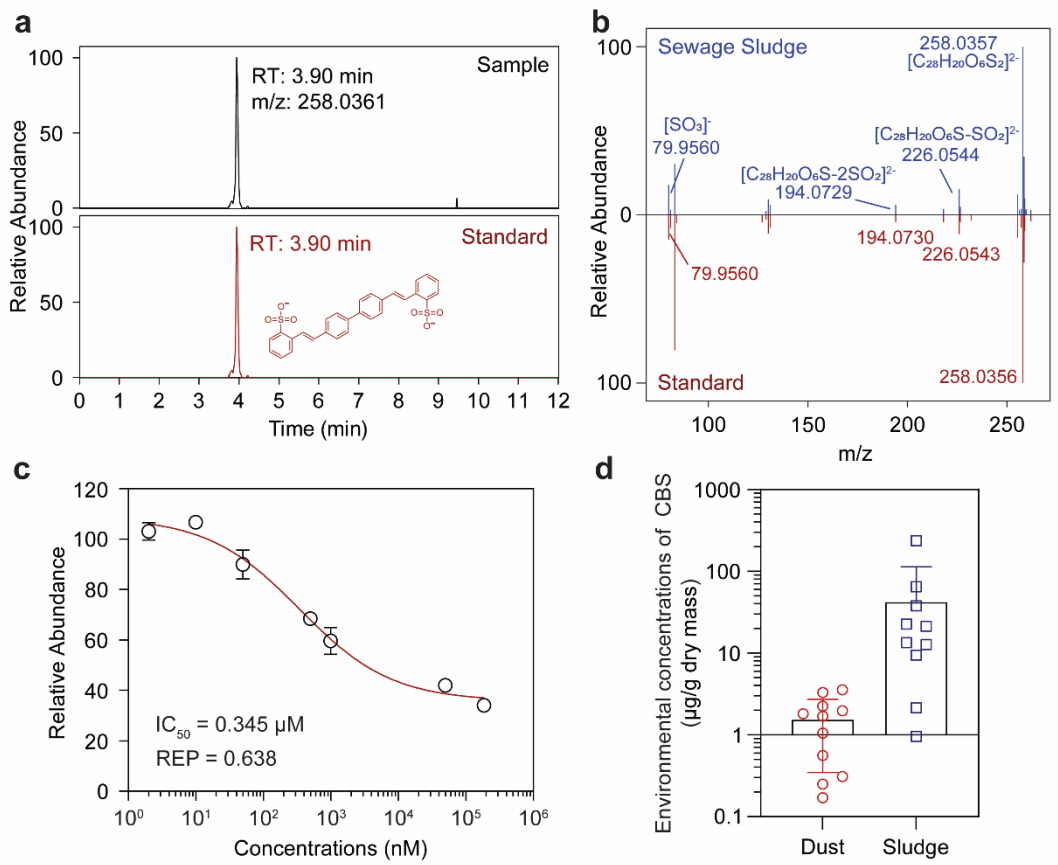

Figure 4. Identification of a previously unrecognized hTTR ligand. (**a**) Liquid chromatograms of CBS from extract of sewage sludge or the authentic standard. (b) MS^2 spectra used to assign the structure of CBS. (**c**) Binding activities of CBS to hTTR determined by the FITC-T_4 displacement assay. $N = 3$. (**d**) Environmental concentrations of CBS in extracts from indoor dust or sewage sludge.

CBS belongs to a class of mass-produced dyestuff chemicals known as fluorescent brighteners (FBs)[56] and has long been widely used as an optical brightener for various detergents. According to the Consumer Product Information Database (CPID, https://www.whatsinproducts.com/), ~158 detergent products contain CBS, and its contents is typically around 0.1-1%.[57] Furthermore, CBS has also been found in one rice noodle product at approximately 2.1 mg/kg in Korea.[58] These results suggested a clear potential for human exposure to CBS. However, the current toxicity information on CBS is surprisingly scarce. A recent study found that exposure to CBS could inhibit the enzyme activity of iodotyrosine deiodinase (IYD), which is an important iodide recycling enzyme for thyroid hormone synthesis.[59] Together with the information regarding its high hTTR binding affinity, IYD inhibition, close human contact, and high

environmental concentrations, CBS might be an important TDC that has been overlooked in previous studies. Future studies are warranted to clarify the potential health effects of exposure to CBS in humans.

Contributions of identified chemicals to total hTTR binding activities. The BEQ concept was used to determine the contributions towards hTTR activity in this study.[60] Four identified hTTR ligands by APNA (i.e., PFOS, dodecyl benzenesulfonate, tetradecyl benzenesulfonate, and CBS) and nine hydrocarbon surfactants which exhibited hTTR binding activities (Table S4) in the FITC-T_4 displacement assay were included. The concentrations of CBS, tetradecyl benzenesulfonate, octyl sulfate, 2-ethylhexyl sulfate, heptanesulfonate, and octanesulfonate in indoor dust and sewage sludge samples were measured by constructing external calibration curves with commercially available standards ($R^2 > 0.99$), while those of the other chemicals were adopted directly from our previous study.[26] Then, by using the REP (Table S4) values and the detected chemical concentrations (Table S5), BEQ_{chem} was calculated (eq 3) for each sample with T_4 as the reference compound. By comparing the BEQ_{bio} and BEQ_{chem} values, the detected chemicals could explain 1.92 to 17.1% (median: 9.95%) and 5.74 to 54.4% (median: 22.5%) of hTTR binding activities in dust and sludge samples, respectively. The contributions of the detected chemicals to the total hTTR effect were shown in Figure 5a and Table S6. Notably, in some samples, the detected chemicals could explain >30% of the hTTR effects (e.g., 54.4% explained in sludge sample S1), which was mostly driven by dodecyl benzenesulfonate and CBS (Figure 5b). This mainly resulted from their extremely high concentrations in the environment (at mg/g level for dodecyl benzenesulfonate)[26] or potent biological activity toward hTTR (REP 0.345 for CBS). In contrast, PFOS, which has been the subject of extensive research attention, only contributed to <0.0001% of the total effects (Table S6). These results were highly intriguing as only 1.2% of hTTR activities could be explained previously by known hTTR ligands in indoor dust.[17] Note that the contributions of hydrocarbon surfactants to hTTR binding activities might be largely underestimated due to the lack of authentic standards for most homologue/isomer compounds. Thus, we concluded

that hydrocarbon surfactants (especially for hydrocarbon sulfonates) and CBS are the major hTTR ligands in the environment.

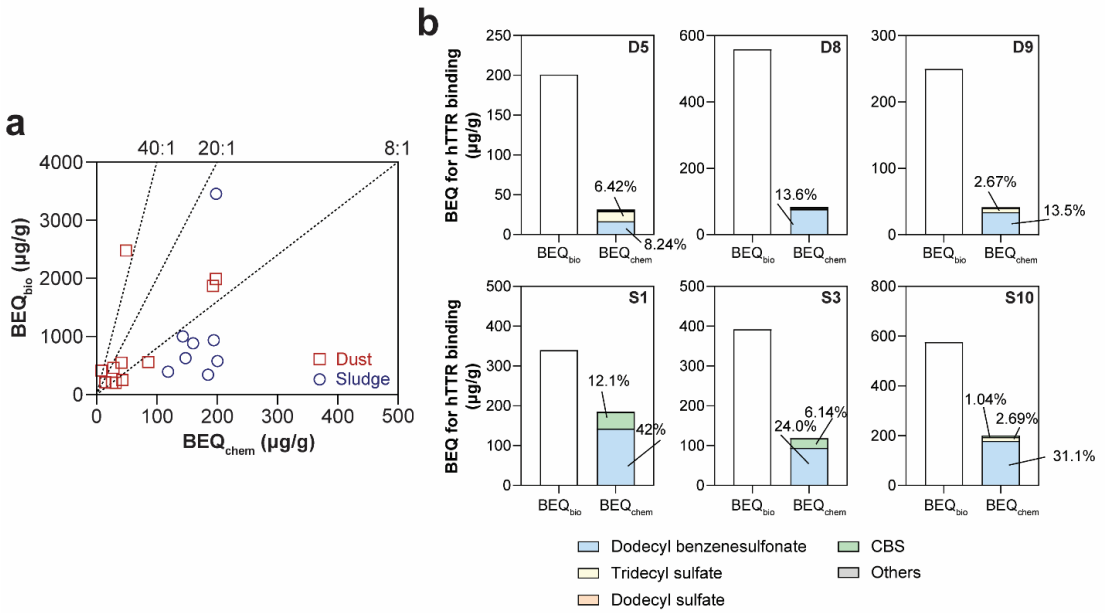

Figure 5. Comparison of biological equivalent concentration from bioanalysis (BEQ_{bio}) and chemical analysis (BEQ_{chem}) for hTTR binding activity (**a**). Red squares and blue circles represent indoor dust and sewage sludge samples, respectively. Contributions of identified hTTR ligands to the total hTTR binding activities in representative indoor dust or sewage sludge samples were shown as bar plots (**b**). D represents indoor dust samples, while S represents sewage sludge samples.

Implications. The incidence of thyroid-related disease including thyroid cancer have rapidly increased over the last several decades.[61] TDCs have long been hypothesized to contribute to this increase, yet known TDCs cannot fully explain the related protein-mediated activities. Indeed, known chemicals have been found to only explain ~1% of the hTTR activity in the environment.[17] Thus, it is important to identify the major TDCs and investigate their potential contribution to thyroid-related disease. In this study, we discovered that hydrocarbon sulfonates, including surfactants and a fluorescent brightener, can explain a large portion of the hTTR activity in the environment. Considering their high production volume, close human contact, and strong hTTR potencies, it is important to investigate the potential health impacts of these two

compound families.

Due to their weak acute toxicities, hydrocarbon surfactants have long been considered 'safe' chemicals, and very limited studies have investigated their chronic toxicities. Recently, they were found to induce stronger toxicity to zebrafish embryo and terrestrial plants than PFAS.[62, 63] In our previous study, we also demonstrated that hydrocarbon surfactants are predominant synthetic ligands for human FABP1 and PPARγ proteins in the environment.[26] Here, we further revealed that hydrocarbon surfactants can also target the hTTR protein (Figure 6), indicating that hydrocarbon surfactants may interfere with multiple biological processes within organisms. Moreover, another European group also independently identified hydrocarbon surfactants as hTTR ligands in treated wastewater and its downstream water, which further validated our results and indicated the wide presence of hydrocarbon surfactants in the environment. A major limitation of the current study is that *in vivo* metabolism and elimination of hydrocarbon sulfonates were not taken into consideration. Considering the close contact of these compounds with humans, *in vivo* animal testing and epidemiological studies are warranted in the future to systematically assess their chemical safety.

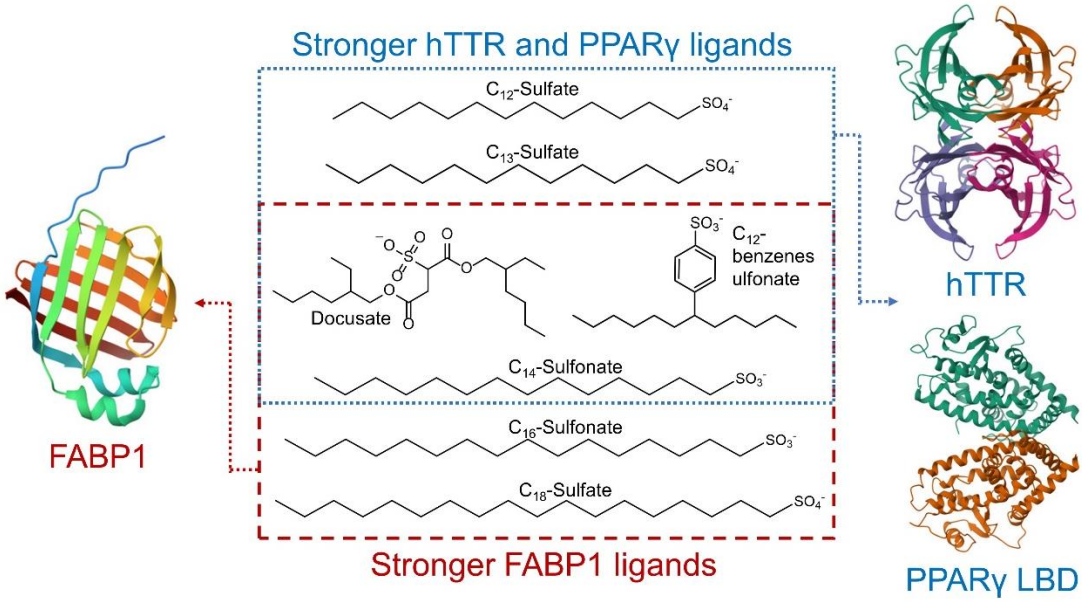

Figure 6. Binding of hydrocarbon surfactants to three human proteins with distinct preference.

Supporting Information Available

The supporting information provides text, tables, and figures addressing: (1) Supplementary materials and methods; (2) Validation of the FITC-T_4 probe; (3) Validation of the displacement assay; (4) hTTR binding activities of indoor dust and sewage sludge samples; (5) Correlation between hTTR and PPARγ LBD activities; (6) Verification of the recombinant His-tagged hTTR protein; (7) Benchmarking of the APNA method; (8) hTTR binding activities of hydrocarbon surfactants; (9) Relationship between carbon chain length and hTTR activity; (10) Molecular docking; (11) Isotopic distributions of the doubly charged ions; (12) List of standards; (13) IC_{50} and BEQ_{bio} values of environmental samples; (14) List of pulled-out LC-MS features; (15) IC_{50} and REP values of tested chemicals; (16) Environmental concentrations of CBS and hydrocarbon surfactants; (17) Contributions of identified chemicals to the total hTTR binding activities.

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