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Competitive Binding of Poly- and Perfluorinated Compounds to the Thyroid Hormone Transport Protein Transthyretin

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Due to their unique surfactant properties, poly- and perfluorinated compounds (PFCs) have been extensively used and can be found all over the environment. Concern about their environmental fate and toxicological properties has initiated several research projects. In the present study, we investigated if PFCs can compete with thyroxine (T_4, i.e., the transport form of thyroid hormone) for binding to the human thyroid hormone transport protein transthyretin (TTR). Such competitive capacity may lead to decreased thyroid hormone levels as previously reported for animals exposed to PFCs. Twenty-four PFCs, together with 6 structurally similar natural fatty acids, were tested for binding capacity in a radioligand-binding assay. The binding potency decreased in the order: perfluorohexane sulfonate > perfluorooctane sulfonate/perfluorooctanoic acid > perfluoroheptanoic acid > sodium perfluoro-1-octanesulfinate > perfluorononanoic acid, with TTR binding potencies 12.5–50 times lower than the natural ligand T_4. Some lower molecular weight compounds with structural similarity to these PFCs were > 100 times less potent than T_4. Simple descriptors based on the two-dimensional molecular structures of the compounds were used to visualize the chemical variation and to model the structure-activity relationship for the competitive potencies of the TTR-binding compounds. The models indicated the dependence on molecular size and functional groups but demanded a more detailed description of the chemical properties and data for validation and further quantitative structure-activity relationship (QSAR) development. Competitive binding of PFCs to TTR, as observed for human TTR in the present study, may explain altered thyroid hormone levels described for PFC-exposed rats and monkeys. Median human blood levels of the most potent TTR-binding PFCs are one to two orders of magnitude lower than concentration at 50% inhibition (IC_{50}) values determined in the present study. In addition, this study contributes to the understanding of the bioaccumulation of PFCs in man and possibly in other wildlife species.

Key Words: **perfluorinated compounds; thyroid hormone; thyroxine; transthyretin binding; perfluorooctane sulfonate; endocrine disruption.**

Poly- and perfluorinated organic compounds (PFCs) are a class of substances characterized by a partially or fully fluorinated alkyl chain and a terminal functional group. The C–F bonds result in great stability under extreme heat and chemical stress and give the compound an oleophobic (oil repelling) property, whereas the polar head contributes to the excellent surfactant property of many PFC (e.g., perfluorinated sulfonates and carboxylates). These unique properties contribute to the widespread use of PFCs in a variety of commercial products, such as household surface finishes, food packaging, water- and stain-resistant materials, fire-fighting foams, etc. (Kissa, 2001).

Environmental research initially focused on the compounds perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), the perfluorinated contaminants predominantly analyzed and also found in the environment. However, a variety of other PFCs are now being found worldwide in the environment, animals, and humans from urban to remote areas in all trophic levels (Houde *et al*., 2006; Kallenborn *et al*., 2004; Kannan *et al*., 2004; Lau *et al*., 2007; van Leeuwen *et al*., 2006).

The structure of many PFCs and their behavior within the body of organisms are comparable to free fatty acids (FAs), and as such they bind to liver FA–binding protein, and the protein albumin, which is mainly present in blood, liver, and eggs (Jones *et al*., 2003; Luebker *et al*., 2002; Martin *et al*., 2003). It is suggested that the polar hydrophobic nature of fluorine-containing compounds can lead to increased affinity for proteins, despite the relatively weak dipolar interactions that characterize the hard C–F dipole. The polar hydrophobic concept can explain some of the protein-binding data characteristics of fluorinated compounds (Biffinger *et al*., 2004). Highest levels of PFCs in rodents, humans, and marine animals are accordingly found in the protein-rich blood and

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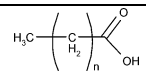
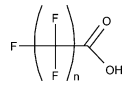
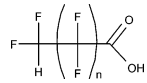
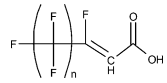
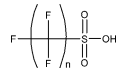
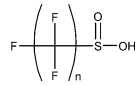
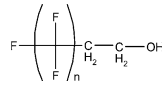
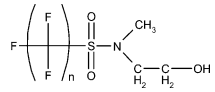
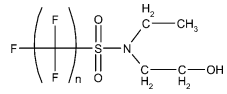
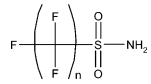
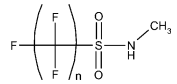
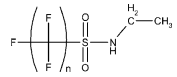
liver (Kannan *et al*., 2004; Kudo and Kawashima, 2003; Lau *et al*., 2003; Luebker *et al*., 2002; Martin *et al*., 2003; Thibodeaux *et al*., 2003; Van den Heuvel *et al*., 1992).

The toxicology of PFCs has recently been extensively reviewed (Kudo and Kawashima, 2003; Lau *et al*., 2007, 2008). Among other observations, decreased thyroid hormone levels after PFC exposure have been found in monkeys and rodents (Lau *et al*., 2003; Luebker *et al*., 2002; Seacat *et al*., 2002, 2003; Thibodeaux *et al*., 2003). It has recently been shown that PFOS does not affect the regulatory functions of the thyroid hormone system itself, but it is the competitive binding to transport proteins that alters the free thyroxine (T_4) levels in blood (Chang *et al*., 2008; Lau *et al*., 2007). By altering thyroid hormone levels, PFCs may affect fetal and neonatal development, especially since PFOS can cross the placental barrier in both humans (Inoue *et al*., 2004) and rodents (Lau *et al*., 2003; Thibodeaux *et al*., 2003). Whether this is caused by diffusion over the membrane or mediated by transport proteins is not yet known. Thyroid hormones are associated (not covalently bound) to transport proteins such as transthyretin (TTR). This complex functions as a circulating reservoir to buffer changes in thyroid hormone levels. TTR is not only a highly conservative plasma protein and the main T_4 carrier in cerebrospinal fluid (CSF) but also important in serum of most mammalian species and birds. TTR is composed of four identical 127 amino acid β sheet–rich subunits. X-ray studies of TTR show two funnel-shaped binding sites for T_4 (Blake *et al*., 1978), each with an inner and an outer binding site. The hydroxyl group as well as the two adjacent iodine molecules on the outer ring of the T_4 structure are binding to the inner binding site of TTR, whereas the iodine atoms on the inner tyrosine ring of T_4 are binding to the outer binding site of TTR (Gosh *et al*., 2000). TTR constitutes ~25% of total CSF proteins (Aldred *et al*., 1995), and the importance of TTR in central nervous system development is evidenced by the fact that it is present in very high concentrations during prenatal and early postnatal life (Larsen and Delallo, 1989).

The aim of this study was to screen the binding capacity of poly- and perfluorinated compounds (PFCs) and structurally similar natural FAs to the thyroid hormone transport protein TTR. Studies on TTR binding of PFCs have not been performed before and may contribute to the understanding of the behavior and toxic mode of action of PFCs, which are used in several applications and are found in many environmental matrices.

The set of PFCs and related compounds selected for testing was based on environmental relevance and on a broad variation in chemical functionalities, i.e., carbon chain length (from 4 to 18 carbons), fluorination degree (from fully to nonfluorinated chains), and different functional groups at the heads of the molecules (carboxylates, sulfonates, sulfonamides, alcohols, etc.). All 30 compound structures are represented in Table 1. In addition, 56 chemical descriptors were calculated to study the structural variation of the PFCs and to evaluate the structure-activity relationship (SAR) of the binding potency to TTR of the PFCs.

TABLE 1
The General Structure of the Compounds Tested in this Study

Natural FA (n = 4–18)	
Perfluorinated alkyl acid (n = 3–13)	
7H-Perfluoroheptanoic acid (n = 5)	
2H-Perfluoro-2-octenoic acid (6:2) (n = 5)	
Perfluorinated alkyl sulfonate (n = 4–10)	
Perfluorinated octane sulfinate (n = 8)	
Perfluorinated telomer alcohol (n = 6–8)	
2-(N-Methylperfluoro-1-octane sulfonamido) ethanol (n = 8)	
2-(N-Ethylperfluoro-1-octane sulfonamido) ethanol (n = 8)	
Perfluorooctane sulfonamide (n = 8)	
N,N-Dimethyl perfluorooctane sulfonamide (n = 8)	
N,N-methyl perfluorooctane sulfonamide (n = 8)	
N-Ethyl perfluorooctane sulfonamide (n = 8)	

MATERIALS AND METHODS

Study compounds. All studied compounds are described in Table 1. For all compounds, 10mM stock solutions were prepared in dimethyl sulfoxide (Acros Organics, Geel, Belgium; 99.9%), except for perfluoroundecanoic acid, perfluorododecanoic acid (PFDoA), and perfluorotetradecanoic acid (PFTdA), where 5mM stock solutions were prepared due to limited solubility at higher concentrations.

TTR-binding assay. The TTR-binding assay has been described in detail earlier (Hamers *et al*., 2006; Lans *et al*., 1993) and is modified from a method first described by Somack *et al*. (1982). Before use, the radiolabeled ^{125}I-labeled T_4 (T_4^*, L-3′-5′-^{125}I-Thyroxine, ≥ 95%; PerkinElmer Life and

Analytical Sciences, Waltham, MA; 4400 Ci/mmol; ~100,000 cpm, ~30pM) was cleaned up to minimize the level of free ^{125}I in the solution due to degradation of T_4^*. The presence of free ^{125}I in the test system leads to an overestimation of free T_4^* levels. Free ^{125}I and T_4^* were separated on a Pasteur pipette column packed with a LH-20 Sephadex (Pharmacia LKB, Woerden, The Netherlands; 0.2 g in 2 ml H_2O, acidified with 3 × 1 ml 0.1M HCl). The ^{125}I activity of each of the following steps can be observed in Supplementary Figure 1. The T_4^* solution was acidified with 1M HCl (1:1, vol/vol), and 0.1M HCl was added to an end volume of 1 ml. T_4^* was added to the column, the tube was rinsed with 1 ml HCl (0.1M), and free iodine was eluted with 3 × 1 ml HCl (0.1M), 3 × 1 ml H_2O, and 0.5 ml ammonia dissolved in ethanol (1:99, vol/vol). To collect the purified T_4^*, ~1 ml ammonia ethanol (1:99, vol/vol) was added, and during constant monitoring of radioactivity, the majority of the T_4^* was eluted from the column in a minimal volume. TTR-binding experiments were performed within 3 weeks of ^{125}I-T_4 cleanup.

The incubation mixture, consisting of human TTR (Sigma-Aldrich, St Louis, MO; 30nM), Tris-HCl buffer (pH 8.0; 0.1M NaCl, 0.1mM EDTA) with a 55nM mixture of T_4^* and unlabeled T_4 (Sigma-Aldrich), and competitor (10–10,000nM), was incubated overnight at 4°C in a final volume of 200 µl. The T_4 levels in the bioassay resemble physiologically relevant T_4 levels close to the lower range of total T_4 in human blood of healthy adults (Hamers *et al*., 2008). Unlabeled T_4 (4–1024nM) was used as a reference compound. After incubation, TTR-bound and free T_4^* were separated on 1-g Biogel P-6PG (Bio-Rad Laboratories, Hercules, CA) columns and spin forced with additional 200 µl Tris-HCl buffer (1 min, 1000 rpm). The TTR-bound T_4^*-containing eluate was counted for radioactivity on a gamma counter (LKB Wallack; 1282 Compu-gamma CS, Turku, Finland) and corrected for the initial amount of T_4^* counted in the incubation mixture before incubation.

Relative T_4^*/T_4-protein binding was plotted against added log competitor concentration, and competition binding curves were least square fitted to the data with nonlinear regression curve (Graphpad Prism, version 5.01). Binding characteristics of the test compounds are calculated as relative potency compared to T_4 (T_4-REP), i.e., the ratio concentration at 50% inhibition (IC_{50}) T_4:IC_{50} test compound. For compounds yielding responses less than 50%, model fits were performed assuming an inhibition slope of −1.38 (mean of high response slopes) and a maximum TTR-binding capacity of 100%.

To exclude that the acidic nature of the PFCs and the FAs affected the pH in the assay, the pH in the incubations was checked with universal indicator paper (Merck, Darmstadt, Germany; resolution 1 pH unit) to monitor any large variations. The pH was shown to be stable at pH 8.

To exclude that a decreased T_4^*-TTR binding was caused by the surfactant character of PFCs, which may change the structure of TTR or decrease the availability of TTR or T_4 in the incubation mixture, additional binding experiments were performed with T_4 (control curve) or a mixture of six PFCs in combination with either 30 or 60nM TTR. The mixture consisted of perfluorobutyric acid (PFBA), perfluorodecanoic acid (PFDcA), PFDoA, PFTdA, 7H-perfluoroheptanoic acid (7H-PFHpA), and PFOS in a molar ratio of 2.7:1.1:1.1:1.1:1.1:1.1:1.0, respectively. The rationale of this experiment was that increasing the TTR concentration leads to an increase in the absolute T_4^*-TTR binding in case of pure competition between T_4^* and PFCs. If PFCs change the structure of TTR or decrease the availability of TTR or T_4 in the incubation mixture, an increase in TTR cannot lead to an increase in TTR-bound T_4^*, given the excess of PFC (maximum 10000nM) in the incubation mixture compared to T_4^* (maximum 1024nM) and to TTR (maximum 60nM).

High Performance Liquid Chromatography (HPLC)-Electron Spray Ionisation (ESI)-tandem Mass Spectrometry (MS/MS). In parallel, retention times were determined for all compounds as a chemical descriptor for SAR evaluation by HPLC-ESI-MS/MS as described by van Leeuwen *et al.* (unpublished data).

Chemical descriptors. Based on two-dimensional molecular structures, chemical descriptors were derived in Molecular Operating Environment (Chemical Computing Group). In total, 56 descriptors were used comprising, e.g., molecular volume, surface area, weight, diameter, radius, number of hydrogen bond donors and acceptors, topological indices such as Zagreb, Balaban, Kier, and Hall chi indices, and partial charges. The partial charge descriptors include 16 parameters reflecting total and fractional positive and negative partial charges and partial charges related to total surface area and hydrophobic and polar surface area. HPLC retention times as described above were also included in the set of chemical descriptors.

SAR analysis. In order to study the chemical diversity of the PFCs, as described by the 56 calculated molecular descriptors (Supplementary Table 1), principal component analysis (PCA) was applied (Jackson, 1991).

A relation between the chemical characteristics of the PFCs and the TTR binding potency was searched using partial least squares regression (PLS). In brief, the systematic variation is searched in an X-matrix, here including the calculated chemical descriptors, and correlated with the systematic variation in the Y-matrix (here IC_{50} values) including the observed response. Latent variables are formed of the matrices and their relationship is maximized through a weight vector. To give each parameter equal chance to influence the model, the data were preprocessed by auto-scaling and mean centering. The dimensionality of the PCA and PLS models was statistically checked by cross-validation (Wold, 1978). For statistical evaluation of the calculated models, R^2X, R^2Y, and Q^2 are given, i.e., the explained variance in the X- and Y-matrix and the cross-validated explained variance, respectively. The predictability of the model can be validated through the Q^2 value or through the value root mean squared error of prediction (RMSEP). RMSEP is calculated using an external validation set, i.e., compounds that were not part of the establishment of the model. RMSEP is calculated as $(PRESS/N)^{1/2}$, where PRESS is the prediction error sum of squares and N equals the number of compounds in the validation set. In order to discuss outlying properties of the compounds' TTR binding potency, DModY was used. DModY is the distance of an observation to the model in the Y space (Eriksson *et al*., 1999). The PCA and PLS calculations were performed on a PC using SIMCA-P version 11.0 software 2005 (Umetrics AB, Umeå, Sweden).

RESULTS

For perfluorinated alkyl acids (PFAA), TTR binding potency varied with carbon chain length (Fig. 1A), with a maximum potency at a chain length of eight carbons (PFOA). The binding potency is clearly associated with the degree of fluorination of the alkyl chain (Fig. 1B). FAs with no fluorine atoms show no binding to TTR (octanoic acid), whereas the fully fluorinated PFOA has a high potency. The 6:2-2H-perfluoro-2-octenoic acid and the 7H-PFHpA with the same number of fluorine atoms ($n = 12$) but different chain lengths (seven and eight, respectively) have similar TTR binding potency. Perfluorohexane sulfonate (PFHxS) has the highest potency (not statistically significant) of the perfluorinated alkyl sulfonates (PFAS), closely followed by PFOS (Fig. 1C). For PFCs with a carbon chain length of four to eight, TTR binding potencies were significantly higher for compounds containing a sulfonate functional group than for those containing a carboxylic acid functional group (paired t-test of mean IC_{50} value in each functional group category, $p = 0.01$). PFAA with a carbon chain length longer than eight have low TTR binding potencies (Fig. 1A), whereas the equivalent PFAS (perfluorodecane sulfonate) exhibits no binding to TTR at all (Fig. 1C). Actually, L-PFDS seemed to increase the % T_4 bound to TTR (Fig. 1C). Similarly, nonfluorinated FAs also exhibit no TTR binding potency at all (Fig. 1D), whereas an increase in T_4-TTR

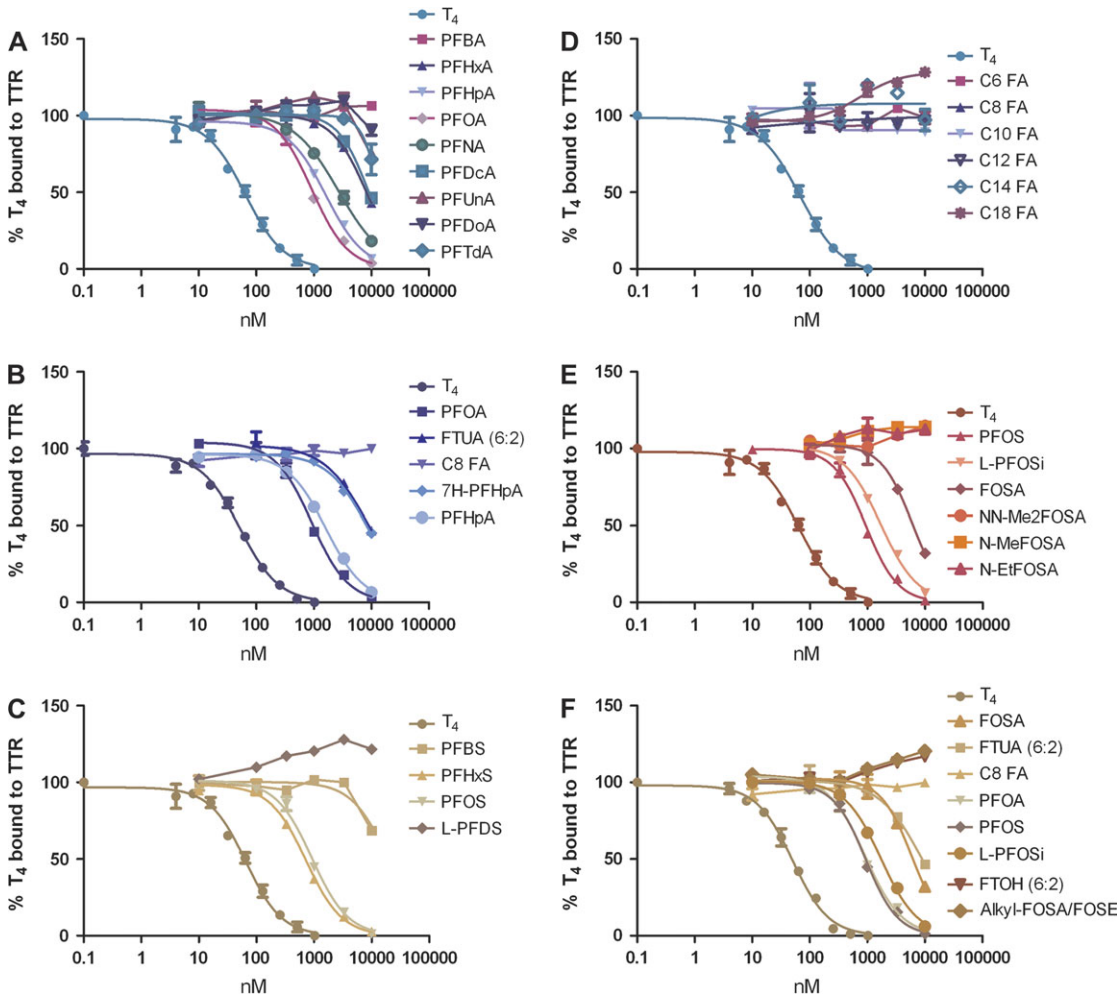


FIG. 1. (A–E) Dose-response curves (% T_4 bound to TTR) for T_4 calibration curves and different concentrations of competitors (nM), i.e., (A) PFAA with different carbon chain length (C4–C14), (B) PFAA with the same carbon chain length (2 × C7 and 3 × C8) and different fluorination grade, (C) PFAS with different carbon chain length (C4–C10), (D) nonfluorinated free FAs with different carbon chain length (C6–C18), (E) PFOS and sulfinate (perfluorinated octane sulfinate) and (N-substituted) perfluorooctane sulfonamides, and (F) PFCs with carbon chain length of eight and different functional groups. Alkyl-FOSA/FOSE represents the curve of N-MeFOSE, N-EtFOSE, N,N-Me2FOSA, N-MeFOSA and N-EtFOSA.

binding was observed at high concentrations of FAs. This phenomenon has earlier been described when Biogel columns were loaded with partially cleaned up environmental samples (Hamers *et al.*, 2008). It is believed that this is not a true increase in TTR binding but rather a methodological artifact by which lipophilic compounds in the sample facilitate the elution of free T_4 through the column.

Of the six PFCs with the same fluorinated carbon chain length (C8) but with different sulfate-based functional groups, highest binding potency was observed for the sulfonate (PFOS), followed by the sulfinate (perfluorinated octane sulfinate) and the sulfonamide (perfluoro-1-octane sulfonamide) (Fig. 1E). Test compounds with the sulfonamide functional group protected by an alkyl group had no TTR binding potency themselves but seemed to cause a slight increase in T_4-TTR binding at high test concentrations (Fig. 1E).

In total, 12 compounds were tested with the same carbon chain length ($n = 8$) but with different number of fluorine on the carbon chain and functional end groups with or without substitutions (Fig. 1F). The binding potency depended on the functional group and the number of fluorine (mentioned in brackets) in the order of SO_3(17) ≈COOH(17) > SO_2(17) > SO_2NH_2(17) > COOH(15) > COOH(0) ≈telomer-OH(13) ≈N-alkyl-SO_2(17) ≈N-alkyl-SO_2NH_2(17).

A summary of all the data derived from the full dose-response curves, i.e., % TTR binding at maximum test concentration, IC_{50}, slope of the curve, T_4-REP, HPLC retention times, as well as the origin and purity of the compounds are given in Table 2. T_4-REP factors ranged from not detected (n.d., $n = 15$) to 0.085 (Fig. 2). The average IC_{50} ± SD of the T_4 calibration curve was 61 ± 5nM ($n = 9$).

This study covers testing of 24 PFCs with a large chemical variation (here reflected by, e.g., molecular volume and area,

TABLE 2

Information about the Analyzed Compounds (Abbreviation, End Functional Group, Full Name, CAS Number, Molecular Weight, Origin of Purchase, and Purity) and A Summary of All the Data Derived from the Full Dose-Response Curves for All Compounds Tested (% Inhibition of T_4-TTR Binding at Maximum Concentration, Concentration at 50% Inhibition (IC_{50}), Slope of Dose-Response Curve, T_4-REP Factor and High Performance liquid Chromatography Retention Time [RT])

Abbreviation	End group	Full name	CAS number	Molecular weight (g/mol)	T_4-TTR binding (%)[a]	IC_{50} (nM)	Slope[b]	T_4-REP factor	RT (min)	Purity (%)
T_4	OH	Thyroxine	7488-70-2	776.9		61	−1.17	1		
C6 FA	COOH	Hexanoic acid	142-62-1	116.2	99	n.d.	—	n.d.	n.a.	≥ 99.5[c]
C8 FA	COOH	Octanoic acid (Caprylic acid)	124-07-2	144.2	100	n.d.	—	n.d.	24.0	≥ 99.5[d]
C10 FA	COOH	Decanoic acid (Capric acid)	334-48-5	172.3	89	n.d.	—	n.d.	34.0	≥ 99.5[d]
C12 FA	COOH	Lauric acid	143-07-7	200.3	97	n.d.	—	n.d.	40.5	≥ 99.5[d]
C14 FA	COOH	Myristic acid	544-63-8	228.4	97	n.d.	—	n.d.	45.3	≥ 99.5[d]
C18 FA	COOH	Stearic acid	57-11-4	284.5	128	n.d.	—	n.d.	51.3	≥ 99.5[d]
PFBA	COOH	Perfluorobutyric acid	375-22-4	214.0	106	n.d.	—	n.d.	11.7	99[e]
PFHxA	COOH	Perfluorohexanoic acid	307-24-4	314.0	43	8220	−1.45	0.007	28.1	≥ 98[e]
PFHpA	COOH	Perfluoroheptanoic acid	375-85-9	364.1	7	1565	−1.26	0.039	31.9	96[f]
PFOA	COOH	Perfluorooctanoic acid	335-67-1	414.0	4	949	−1.41	0.064	34.7	96[f]
PFNA	COOH	Perfluorononanoic acid	375-95-1	464.0	18	2737	−1.11	0.022	37.1	97[c]
PFDcA	COOH	Perfluorodecanoic acid	335-76-2	514.0	46	8954	−1.65	0.007	39.1	≥ 98[e]
PFUnA	COOH	Perfluoroundecanoic acid	2058-94-8	563.9	74	21,560	(−1.38)	0.003	40.9	95[c]
PFDoA	COOH	Perfluorododecanoic acid	307-55-1	614.0	91	46,894	(−1.38)	0.001	42.4	95[c]
PFTdA	COOH	Perfluorotetradecanoic acid	376-06-7	713.9	71	28,996	(−1.38)	0.002	45.0	96[e]
7H-PFHpA	COOH	7H-Perfluoroheptanoic acid	1546-95-8	346.1	45	8637	−1.19	0.007	n.a.	98[e]
FTUA (6:2)	COOH	2H-Perfluoro-2-octenoic acid (6:2)	n.a.	358.1	47	8848	−1.18	0.007	30.0	> 99[g]
PFBS	SO_3	Perfluorobutane sulfonate	2795-39-3	300.0	69	19,460	(−1.38)	0.003	24.2	n.v.[c]
PFHxS	SO_3	Perfluorohexane sulfonate	3871-99-6	400.0	3	717	−1.40	0.085	32.3	≥ 98[d]
PFOS	SO_3	Perfluorooctane sulfonate	2795-39-3	500.0	1	940	−1.56	0.065	37.2	≥ 98[d]
L-PFDS	SO_3	Perfluorodecane sulfonate	n.a.	622.1	122	n.d.	—	n.d.	40.0	> 99[g]
L-PFOSi	SO_2	Perfluorooctane sulfinate	n.a.	506.1	6	1733	−1.38	0.035	38.0	> 99[g]
FTOH (6:2)	OH	2-Perfluorohexyl ethanol	647-42-7	364.1	117	n.d.	—	n.d.	34.4	98[e]
FTOH (8:2)	OH	2-Perfluorooctyl ethanol	678-39-7	464.1	117	n.d.	—	n.d.	n.a.	98[e]
N-MeFOSE	NR-(CH_2) OH	2-(N-methylperfluoro-1-octane sulfonamido) ethanol	24448-09-7	557.2	119	n.d.	—	n.d.	46.1	> 99[g]
N-EtFOSE	NR-(CH_2) 2OH	2-(N-ethylperfluoro-1-octane sulfonamido) ethanol	1691-99-2	571.3	122	n.d	—	n.d.	47.1	> 99[g]
FOSA	NH_2	Perfluorooctane sulfonamide	754-91-6	499.2	32	6124	−1.63	0.010	43.1	> 99[g]
N,N-Me2FOSA	NR_2	N,N-dimethyl perfluorooctane sulfonamide	n.a.	527.2	115	n.d.	—	n.d.	n.a.	> 99[g]
N-MeFOSA	NRH	N-methyl perfluorooctane sulfonamide	31506-32-8	513.2	114	n.d.	—	n.d.	46.1	> 99[g]
N-EtFOSA	NRH	N-ethyl perfluorooctane sulfonamide	4151-50-2	527.2	112	n.d.	—	n.d.	47.1	> 99[g]

n.a., not available; n.d., not detected; n.v., not verified.

[a]At maximum concentration (10μM) of competitor.

[b]The mean slope of the dose-response curves reaching 50% binding are given in brackets.

[c]Sigma-Aldrich Chemie GmbH, Buchs, Switzerland.

[d]Fluka, Buchs, Switzerland.

[e]ABCR GmbH & Co, Karlsruhe, Germany.

[f]Acros Organics.

[g]Wellington Laboratories, Guelph, Canada.

connectivity indices, retention times, etc.) as illustrated in the PCA score plot describing 83% of the chemical variation with two principal components (Fig. 3). The PCA based on 56 calculated chemical descriptors revealed clear groupings among the compounds. The most significant chemical property that separates the chemicals in the first dimension (t1) of the PCA is the molecular size, here reflected by, e.g., molecular volume and area, connectivity indices, retention times, and

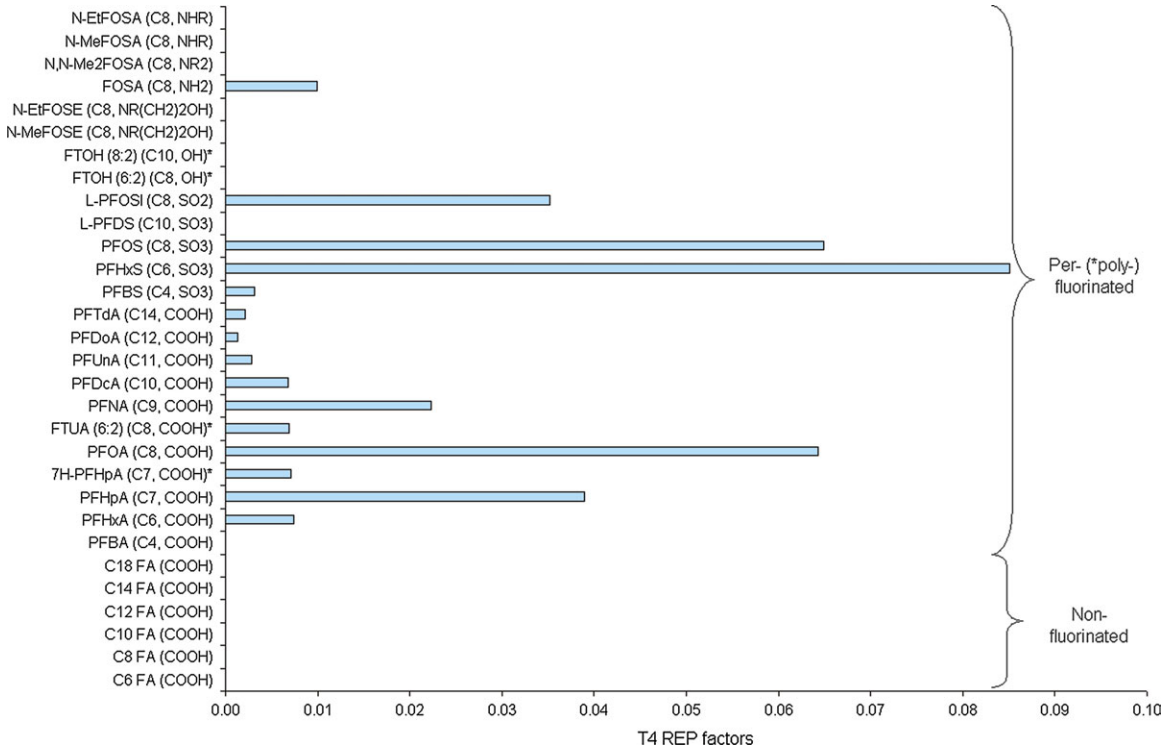


FIG. 2. The results of the TTR binding assay expressed as T4 equivalent factors for all tested compounds. The carbon chain length and the functional end group are given between brackets.

total positive partial charge. Large compounds (e.g., PFTdA) have high score values in the t1, and small compounds, such as PFBA and perfluorobutane sulfonate (PFBS), have low score values. PFBS and PFBA are also clearly separated from their respective chemical class based on their short chain length. The acids and telomer alcohols are split from the sulfonamides and sulfonates based merely on their partial charge characteristics (second dimension [t2]). These compounds have lower total

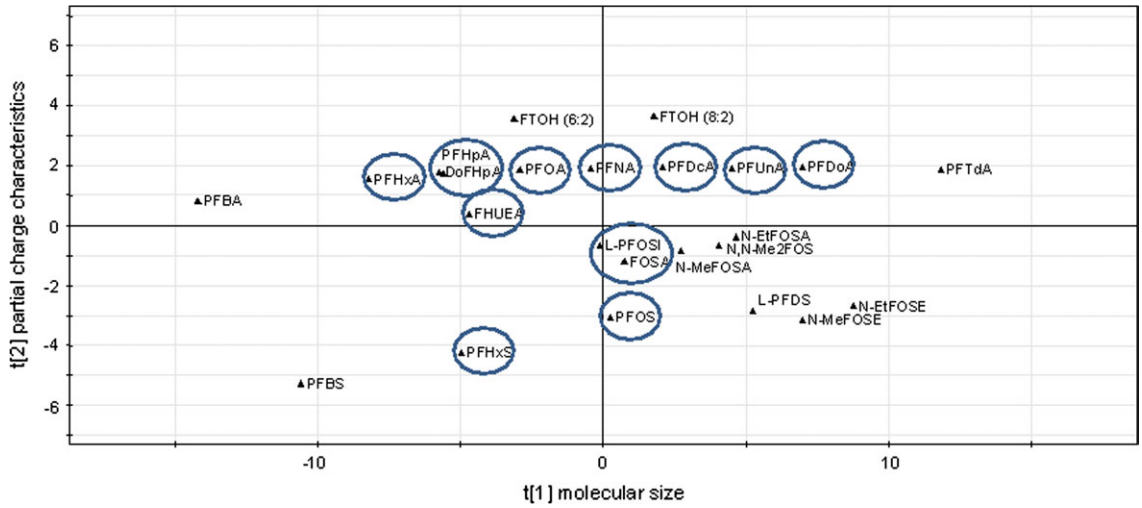


FIG. 3. Score plot with the first two dimensions (t1 and t2) where all tested fluorinated compounds were characterized using 56 chemical descriptors. Encircled compounds are those with relative TTR binding potencies; IC_{50} values < 10μM. The corresponding loading plot is given in the supplementary material. The compounds are abbreviated according to Table 2. The carbon chain length and the functional end group is given between brackets.

and relative negative polar surface area and higher relative hydrophobic surface area (Supplementary Fig. 2). The amides and sulfonates are less well separated in the t2.

Those chemicals with IC_{50} < 10μM are marked in the score plot in Figure 3. Clearly, the chemical domain of the most active substances covers average size PFCs and mainly the acids. Associations were searched between chemical properties and TTR-binding potencies of the PFCs to establish a quantitative structure-activity relationship (QSAR) model and to increase our understanding of the mechanism of action involved. However, the resulting PLS model developed on the 56 chemical descriptors and the IC_{50} values for all 15 active PFCs were not significant. The PLS model diagnosed PFBS as an outlier according to its distance to the X- and the Y-block (DModX and -Y), i.e., according to its chemical properties and its TTR binding potency. In a second PLS model excluding PFBS, two significant components were found explaining 61% of the variation in the response using 88% of the chemical variation and with a cross-validated explained variance of 41%. The observed versus predicted IC_{50} values plot is shown in Figure 4. Significant chemical descriptors in this model were size-related connectivity indices and diameter and radius, and surface-describing descriptors related to total and negative polar surface area.

Under standard testing conditions, the PFC mixture consisting of PFBA, PFDcA, PFDoA, PFTdA, 7H-PFHpA, and PFOS (Fig. 5) had an IC_{50} value of 511nM (SD 11), expressed as PFOS concentration in the mixture. Doubling the TTR concentration (60nM) yielded a dose-response curve with a maximum T_4-TTR binding of 202%, while the IC_{50} of the PFC mixture did not differ significantly (510nM, SD 25; *F*-test; $p > 0.05$). This indicates that the decreased T_4-TTR binding in the presence of PFCs is caused by true competition between T_4 and PFCs and not by changes in T_4 or TTR availability due to the surfactant character of PFCs.

To evaluate whether the TTR binding potencies of PFCs are additive or not, test concentrations of the PFC mixture were expressed as T_4-equivalent (T_4-EQ) concentrations. First, the concentration of each individual PFC in the mixture was multiplied with its respective T_4-REP factor. Next, the T_4-EQ concentrations of the six individual PFCs were summed up into a single T_4-EQ value for the mixture. The measured TTR binding potency of the PFC mixture was plotted against the T_4-EQ concentration (Fig. 6) and could be compared directly to the T_4 calibration curve since concentrations of T_4 and PFCs have similar units (i.e., nM T_4-EQ). Compared to the T_4 curve, the dose-response curve of the PFC mixture was significantly shifted to the left (*F*-test; $p < 0.01$), yielding an IC_{50} for the PFC mixture (37nM T_4-EQ, SD 2) that was 1.6 times lower than the IC_{50} of T_4 (61nM T_4-EQ, SD 5). This result indicates that less PFC is needed for a 50% inhibition of T_4-TTR binding than was to be expected based on concentration addition. It is not to be expected that the statistically significant 1.6-fold deviation from concentration addition has high biological significance. Nevertheless, the possible synergistic effect of PFCs deserves further investigation with different PFC mixtures in different biologically relevant compositions reflecting exposure profiles of vulnerable groups as children, toddlers, pregnant women, and occupationally exposed humans.

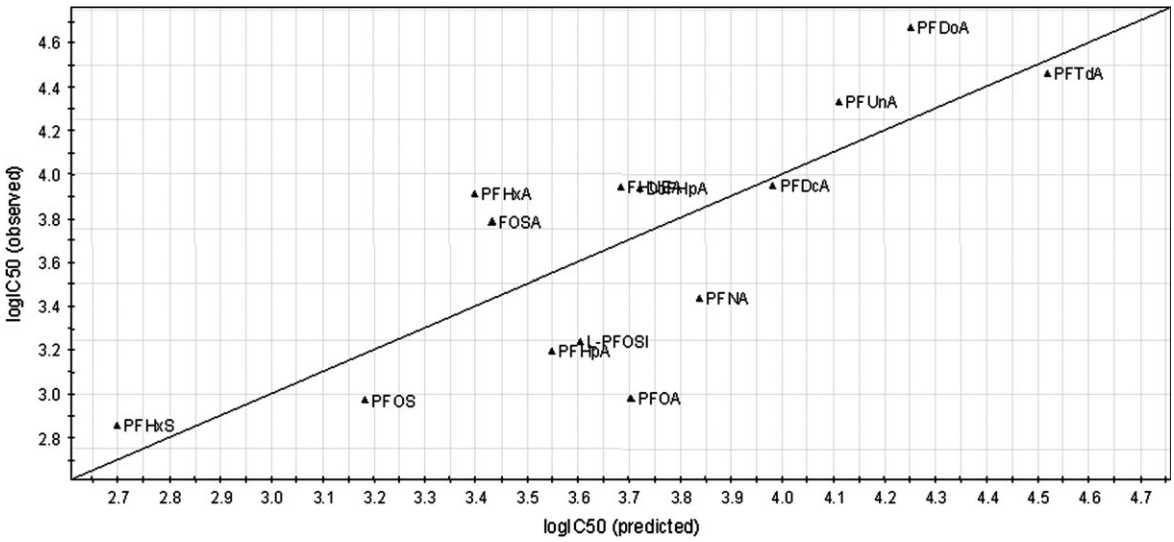


FIG. 4. Observed versus predicted relative TTR binding potencies (IC_{50}) for 23 polyfluorinated compounds calculated using PLS. The responses were normalized by the logarithm prior modeling. The compounds are abbreviated according to Table 2.

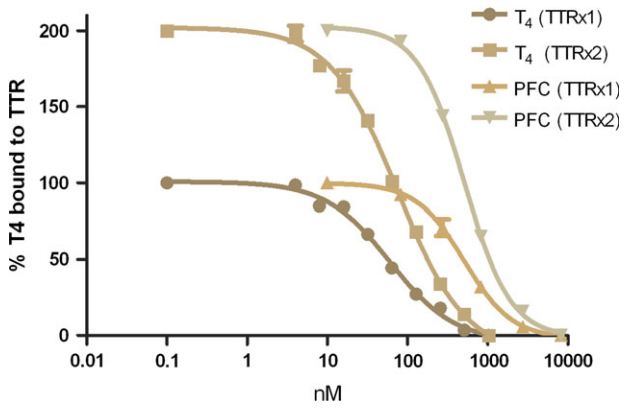


FIG. 5. Dose-response curves (% T_4 bound to TTR) for T_4 calibration curves and different concentrations of a PFC mixture (nM) with two different concentrations of TTR.

DISCUSSION AND CONCLUSION

All three chemical functionalities that varied (i.e., degree of fluorination, carbon chain length, and functional end group) in the test set affected the TTR binding potency of PFCs. PFCs with higher fluorination degree show higher affinity for TTR compared to lower fluorinated PFCs, which is similar to earlier studies with halogenated phenols (Gosh *et al*., 2000; Meerts *et al*., 2000; Ucán-Marín *et al*., 2009; van den Berg, 1990). The length of the PFC carbon chain has been reported to affect several properties, e.g., the bioaccumulation factor (BAF) and the depuration rate constant of PFAA and PFAS in rainbow trout (Martin *et al*., 2003), and the potency of PFOS to inhibit gap junctional intercellular communication in rats and dolphins (Hu *et al*., 2002). The two latter effects peaked around a chain length of eight carbons, whereas the BAF for PFAA increased with a chain length of more than eight carbons; PFAS and PFAA with chain lengths shorter than seven and six carbons, respectively, could not be detected in most tissues and were considered to have insignificant BAFs.

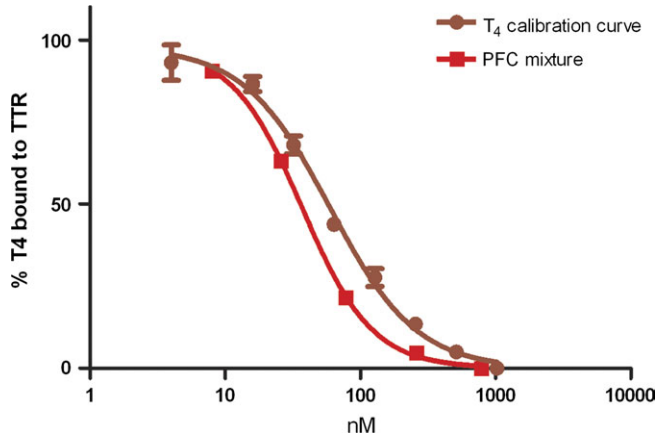


FIG. 6. Dose-response curves (% T_4 bound to TTR) for T_4 calibration curve and a theoretical curve of the PFC mixture based on the individual determined T_4-REP factors.

The functional group is known to change the affinity for TTR in an amyloid fibril inhibition assay (Baures *et al*., 1998), where 78 compounds from different chemical classes were tested. The nitro substituents were less effective compared to carboxylates, while compounds with a hydroxyl group on the aromatic skeleton had the highest affinity. The hydroxyl group at the *para* or *meta* positions in the phenolic compounds significantly increased the binding potency to TTR (Gosh *et al*., 2000; Hamers *et al*., 2008; Meerts *et al*., 2000; van den Berg, 1990). Apparently, the hydroxyl group itself without attachment to an electrophilic aromatic system is not enough to bind to the TTR, as we found no TTR binding by PFCs with functional hydroxyl groups. The importance of the functional group is also demonstrated by the lack of TTR binding observed for the N-substituted perfluoroalkyl sulfonamides. Apparently, acidic PFCs have much higher TTR binding potency than nonacidic PFCs. In our test system (pH = 8), acidic PFAS and PFAA (pKa < 3) are dissociated, while nonacidic fluorotelomer alcohols with much higher pKa values are nondissociated. Hence, the anionic state of acidic PFCs seems to enhance the TTR binding potency.

The hydroxyl group of the natural TTR ligand T_4, which is accompanied by two adjacent iodine atoms, is oriented to the inner part of the TTR-binding site (Gosh *et al*., 2000). An alternative mechanism of binding to TTR has been shown, where the hydroxyl group of the potent TTR-binding compounds can be directed to the outer binding site, emphasizing that the presence of hydroxyl groups is not essential to be able to bind to TTR (Baures *et al*., 1998; Gosh *et al*., 2000). This "reversed" orientation was the exclusive binding mode for penta- and tri-bromophenols (Gosh *et al*., 2000).

An x-ray crystallography study demonstrated that the anti-inflammatory drug flufenamic acid binds to TTR by occupying the innermost halogen-binding pocket (Peterson *et al*., 1998). The CF_3 substituent on the phenyl ring of flufenamic acid interacts with Van der Waals bonds to the amino acids Ser-117, Thr-119, Leu-110, and Ala-108. The carboxylate group on the other phenyl ring of flufenamic acid is located near the entrance of the binding site, forming electrostatic interactions with the side chains of the Lys-15 residues from opposing TTR subunits. The reversed binding orientation might be in accordance with the PFCs tested in this study, but this remains to be verified by structural methods.

The levels of the fluorinated compounds detected in blood of animals and humans are due to a strong association of the PFCs to proteins. The main carrier is believed to be albumin due to its high affinity for PFCs and high concentration in serum (Jones *et al*., 2003). Several other serum proteins have been shown to be associated to PFCs in serum, e.g., sex hormone–binding globulin, corticosteroid-binding globulin, and liver FA–binding protein (Jones *et al*., 2003; Luebker *et al*., 2002;

Van den Heuvel *et al*., 1992). The present study clearly demonstrates that TTR binding of PFCs can also explain the mechanism of PFC retention in human blood. Possibly, the same mechanism can explain the retention of PFCs in other species, as observed in laboratory studies and in wildlife (Houde *et al*., 2006; Kudo and Kawashima, 2003; Lau *et al*., 2007; Luebker *et al*., 2002). It should be realized, however, that structural differences in TTR exist between species leading to different binding affinities. Although human TTR shows high similarity to TTR from other mammalian species such as rat (Cody, 2002) and monkey (Van Jaarsveld *et al*., 1973), human TTR–binding affinity of endocrine disrupting compounds differs from TTR-binding affinity in fish (Morgado *et al*., 2007) and birds (Ucán-Marín *et al*., 2009).

It has been indicated that PFCs can be associated to blood cells in whole blood (Kärrman *et al*., 2006), although a contradictory study states that the PFCs are only found intracellularly (Ehresman *et al*., 2007).

Human exposure levels of PFCs have recently been extensively reviewed (Fromme *et al*., in press). Median plasma or serum levels of PFOS, PFOA, and PFHxS in human adults are 30, 12, and 4nM, respectively, in Europe ($n = 7$ studies), and 60, 11, and 6nM, respectively, in North America ($n = 13$). Comparing these levels to the IC_{50} values for determined in the present study (Table 2) shows that blood levels of the most potent TTR-binding PFCs are 23–153 times below IC_{50} level in European adults and 16–125 in North American adults. Alternatively, these blood levels can be converted into T_4-EQ concentrations corresponding to 3.1 and 5.1nM T_4 in European and North American adults, respectively. In pregnant rats exposed to PFOS from gestation day (GD) 2–20, decreased levels of total serum T_4 were found on GD 7 in the lowest dose group (1 mg/kg/day), corresponding to maternal serum levels of 12 μg/ml PFOS (Thibodeaux *et al*., 2003). Based on a molecular weight of 500 g/mol and a T_4-REP of 0.065 for PFOS (Table 2), this lowest observed adverse effect level (LOAEL) can be recalculated into 1.56μM T_4-EQ. Comparing the LOAEL to the PFC-related median T_4-EQ levels in serum from European and North American adults suggests a margin of safety (MOS) of 503 and 306, respectively.

This ratio between PFC blood levels and the IC_{50}-value for TTR-binding, as well as the calculated MOS based on T_4-EQ concentrations are relatively small, especially when we consider the fact that the latter does not account for uncertainty factors for interspecies and intraspecies differences and for conversion of the LOAEL into a no observed adverse effect level. Although TTR is less important for thyroid hormone transport in humans than in rodents, TTR is both in humans and in rodents the most important carrier protein for thyroid hormone to the developing fetus and the brain. Intraspecies differences are relevant because the MOS calculation is based on median PFC levels in serum, while in most studies, the range of PFC levels in adult serum spans at least one order of magnitude (Fromme *et al*., in press). Vulnerable groups, such as hypothyroid children and pregnant women, may also be more at risk. In addition, humans are not only exposed to PFCs but also to other halogenated environmental pollutants with significant TTR binding potencies, such as pentachlorophenol, tetrabrominated bisphenol-A, and metabolites of polychlorinated biphenyls and polybrominated diphenylethers with T_4-REP factors > 1(Hamers *et al*., 2006, 2008; Lans *et al*., 1993; Meerts *et al*., 2000).

Finally, calculating the TTR binding potency of a mixture of PFCs into a T_4-EQ concentration may underestimate the actual combined potency of the mixture. In fact, the underlying concept of concentration addition does not seem to be valid, given the results from the mixture study indicating that the T_4-EQ potency of a PFC mixture is synergistic.

The median T_4-EQ concentrations of PFCs in human serum can also be directly compared to the natural level of T_4 in serum from healthy adults, i.e., 64–154nM (www.thyroidmanager.org/). The ratio between the natural T_4 levels and the anthropogenic T_4-EQ level attributable to PFCs is 21–50 and 13–30 times for European and North American adults, respectively, suggesting that these PFC levels could be able to interfere with the natural thyroid hormone serum transport.

A considerable lack of experimental data on the physicochemical parameters of PFCs hinders the construction of (Q)SARs. Chemical descriptors for these chemicals are difficult to estimate, as was recently shown for the octanol-water partition coefficient, vapor pressure, and air-water partition coefficient (Arp *et al*., 2006). The values of these parameters were shown to vary considerably depending on the applied method. We also noted variations of up to five orders of magnitude for logP as calculated using five different logP methods (data not shown).

In the present work, we used simple descriptors based on the two-dimensional molecular structure of the compounds to visualize their chemical variation and for an attempt to model the SAR for the potency of PFCs to bind to TTR. The models indicate the dependence on molecular size and functional group but warrant a more detailed description on the chemical properties and data for validation and development.

In conclusion, the results of our study contribute to the understanding of the decreased levels of free T_4 found in the presence of PFCs in serum and of the bioaccumulation of PFCs in humans and wildlife that may be partly attributed to the affinity of PFCs to bind to TTR in serum. PFCs have a TTR binding potency of only one-tenth of the natural hormone T_4, and they are less potent than many other environmental pollutants. However, due to their relatively high concentrations found in the environment (i.e., foremost PFOS) PFCs might contribute to the thyroid hormone–disrupting risk of environmental pollutants. The results also support the observations that binding to TTR can take place even in the absence of a hydrogen bond interaction of the hydroxyl group with the Ser-117 residue of the TTR. Due to earlier findings and because of the structural similarity with the natural ligand T_4,

the research has so far been focused on phenolic compounds regarding the thyroid hormone disruption. This study suggests that a more diverse and larger number of halogenated compounds present in the environment may have the potential to inhibit and hence adversely affect the thyroid hormone system in animals and humans.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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