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Biosensor discovery of thyroxine transport disrupting chemicals

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ABSTRACT

Ubiquitous chemicals may interfere with the thyroid system that is essential in the development and physiology of vertebrates. We applied a surface plasmon resonance (SPR) biosensor-based screening method for the fast screening of chemicals with thyroxine (T4) transport disrupting activity. Two inhibition assays using the main thyroid hormone transport proteins, T4 binding globulin (TBG) and transthyretin (TTR), in combination with a T4-coated biosensor chip were optimized and automated for screening chemical libraries. The transport protein-based biosensor assays were rapid, high throughput and bioeffect-related. A library of 62 chemicals including the natural hormones, polychlorinated biphenyls (PCBs), polybrominated diphenylethers (PBDEs) and metabolites, halogenated bisphenol A (BPA), halogenated phenols, pharmaceuticals, pesticides and other potential environmentally relevant chemicals was tested with the two assays. We discovered ten new active compounds with moderate to high affinity for TBG with the TBG assay. Strikingly, the most potent binding was observed with hydroxylated metabolites of the brominated diphenyl ethers (BDEs) BDE 47, BDE 49 and BDE 99, that are commonly found in human plasma. The TTR assay confirmed the activity of previously identified hydroxylated metabolites of PCBs and PBDEs, halogenated BPA and genistein. These results show that the hydroxylated metabolites of the ubiquitous PBDEs not only target the T4 transport at the TTR level, but also, and to a great extent, at the TBG level where most of the T4 in humans is circulating. The optimized SPR biosensor-based transport protein assay is a suitable method for high throughput screening of large libraries for potential thyroid hormone disrupting compounds.

Introduction

Thyroid hormones (TH), such as L-thyroxine (T4) and its biologically active metabolite, triiodothyronine (T3), are essential for the modulation of the cellular metabolic rate and for the development, and differentiation of several tissues, specially the brain (Yen, 2001; Bernal et al., 2003). The thyroid gland produces and releases T4 in response to the thyroid-stimulating hormone released by the pituitary. Once T4 enters the blood stream, it is bound to transport proteins and distributed to the target tissues. Thyroxine binding globulin (TBG) is the major T4 transport protein in human plasma and is responsible for 75% of the specific T4 binding activity. Transthyretin (TTR) is the second transport protein carrying about 20% of T4 and finally, human serum albumin (HSA) is the third transport protein carrying about 5% of the total T4. The affinity of the three transport proteins for T4 varies greatly, as well as their plasmatic concentration (0.2 μM for TBG, 5 μM for TTR and 600 μM for HSA) providing a redundant buffer system for free T4 (Schreiber, 2002). T4 is specifically transported through the cell membrane into the intracellular compartment and deiodinated to the biologically active T3 metabolite in different tissues (Friesema et al., 2005). Once inside the cell, T3 is non-specifically transported to the nucleus where it exerts its action by binding to the nuclear thyroid receptors. Several chemical contaminants resembling T4 have been shown to interfere at different levels with the thyroid pathway (for review see (Boas et al., 2006)). The disruption of the T4 transport has received special attention since several polyhalogenated aromatic hydrocarbons (PHAHs) become bioactivated in vivo upon hydroxylation by the cytochrome P450 monooxygenase system (Hakk and Letcher, 2003). These hydroxylated PHAHs interact with TTR (Lans et al., 1993; Hallgren and Darnerud, 2002) and some of them, albeit with much lower affinity, interact with TBG (Cheek et al., 1993; Lans et al., 1994). Some studies exclude the dependence of thyroid hormone homeostasis on any transport protein per se (Palha, 2002). However, TTR is important for maternal to fetal transport of thyroid hormones and for delivery of T4 across the brain

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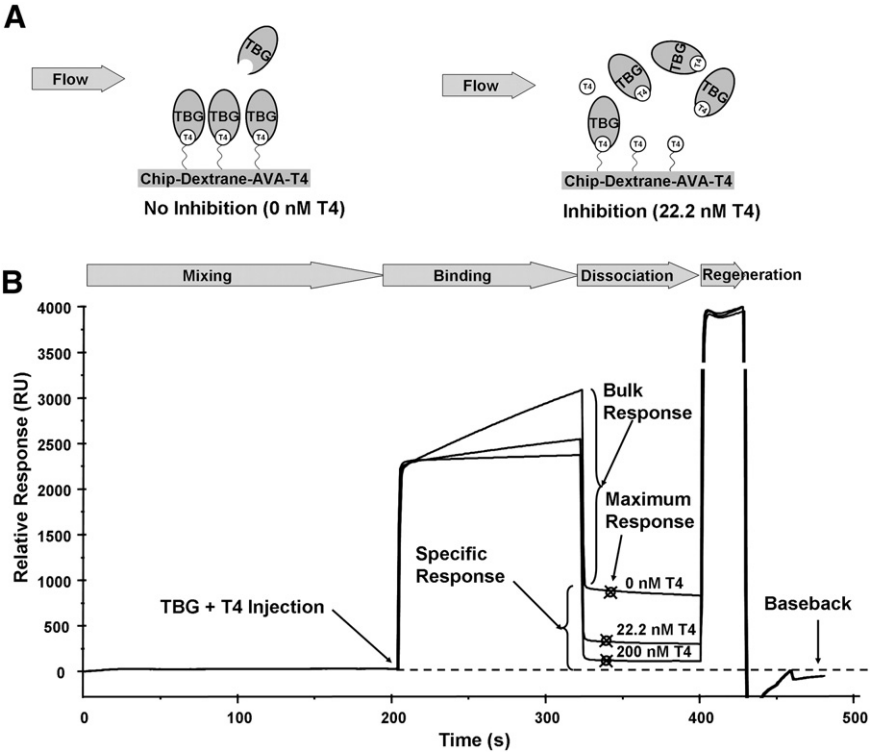


Fig. 1. (A) Principle of the biosensor transport protein-based inhibition assay (assay). (B) Overlayed sensorgrams showing the responses obtained in time during a whole SPR analysis cycle (mixing, injection (binding), dissociation and regeneration) and the inhibition of TBG binding to the T4 chip surface when mixed with 0, 22.2 and 200 nM of T4.

barrier (Schreiber, 2002). TBG has been linked to facilitate the iodine supply to the fetus that initially has no iodine reserve (Schussler, 2000). Hence, it is expected that a displacement of T4 from its transport protein complex during the developmental stage could have consequences in fetal development and later in adulthood (Koopman-Esseboom et al., 1994; Morse et al., 1996). For instance, disturbances in the retinoid and TH levels by environmental chemicals during development has been linked with effects on brain development (Porterfield, 2000) and with lower IQ levels in adulthood (Fritsche et al., 2005). Moreover, obesity is increasingly been linked to environmental chemicals and this could be mediated through an impaired TH homeostasis (Grun and Blumberg, 2006; Tabb and Blumberg, 2006; Newbold et al., 2007). Therefore, the presence and bioaccumulation of these bioactive contaminants in the food chain and finally in humans is a cause of concern. PCBs have been shown to interfere with the TH homeostasis, however, the PCBs concentration in human tissues is gradually declining due to the regulations imposed in the 1970s (Sjodin et al., 2004). On the contrary, the widely used polybrominated diphenyl ethers (PBDEs) flame retardants that were later introduced, have been measured in human adipose tissues from inhabitants of the United States at concentrations of 35 ng/g lipid. This is 10–100 times greater than those reported in European countries and their concentrations have been increasing exponentially for the last 30 years, with a doubling period of about 5 years (Hites, 2004; Sjodin et al., 2004). Although the influence of PBDEs on human T4 levels is unknown, one epidemiological study found a significant negative correlation between thyroid-stimulating hormone and BDE 47 levels in Scandinavian men (Boas et al., 2006).

As a consequence, there is a rising need for high throughput screening methods to determine whether chemicals are affecting the thyroid system at different levels. In the present study we used two transport protein-based surface plasmon resonance (SPR) biosensor inhibition assays (Marchesini et al., 2006). The principle of these biosensor assays is similar to an inhibition immunoassay (see Fig. 1A). If there is no binding of the analyte to the transport protein, the maximum amount of transport protein will bind to the sensor surface and a maximum response is obtained. The binding of transport protein to the sensor surface will be inhibited given an interaction between the analyte and the transport protein in solution, resulting in a lower response. The inhibition of the response depends on the concentration of the T4-like analyte as well as on the affinity of this compound for the transport protein.

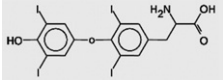
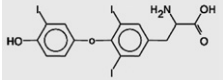
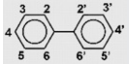
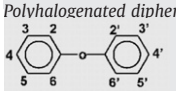
The biosensor assays were optimized to endure long periods of automated operation and used to screen a library of 62 chemicals for compounds affecting the T4 transport. The library, shown in Tables 1 and 2, included the thyroid hormones, polyhalogenated biphenyls, polyhalogenated diphenyl ethers and metabolites, bisphenol A (BPA) and synthetic halogenated derivatives, pharmaceuticals, pesticides and other environmentally relevant chemicals covering a wide range of structural classes.

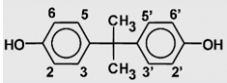
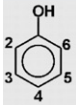
Materials and methods

Chemicals and equipment. The recombinant transthyretin (rTTR) was produced at the Toyama Medical and Pharmaceutical University (Toyama, Japan) (Matsubara et al., 2003). Chemical standards (Tables 1 and 2) had a purity above 95% and were obtained from AccuStandard (New Haven, CT), LGC Promochem (Teddington, UK), Honeywell Riedel de Haën (Seelze, Germany) or Dr. Ehrenstorfer GMbH (Augsburg, Germany). Thyroxine binding globulin (TBG) was obtained from Scipac (Kent, UK). CM5 sensor chips, the amine coupling kit [containing 0.1 M N-hydroxysuccinimide (NHS), 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1 M ethanolamine hydrochloride (pH 8.5)] and the Biacore Q biosensor were supplied by GE Healthcare (Uppsala, Sweden). The 5-aminovaleric acid (AVA), L-thyroxine and the ovalbumin (OVA) and all other reagents were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands) unless otherwise stated.

Transport protein inhibition assays. The transport protein assays, of which the principle is shown in Fig. 1A, were performed as described

Table 1
Data set of polyhalogenated aromatic hydrocarbons (PHAHs) obtained with the biosensor transport protein inhibition assays

Compound	Abbreviation	Source	CAS	TTR						TBG					
				PI (%)	HTC (μM)	IC_{50} (nM)	RP	Category[a]	Literature RP	PI (%)	HTC (μM)	IC_{50} (nM)	RP	Category	Literature RP
Thyroid hormones															
Thyroxine 	T4	S-A[b]	51-48-9	94	0.2	18.4	1	StB		91	0.2	17.2	1	StB	
Triiodothyronine 	T3		6893-02-3	86	1.8	419	0.04	MB	0.02 (Rickenbacher et al., 1986) 0.08 (Somack et al., 1982) 0.09 (Andrea et al., 1980) 0.08 (Cheng et al., 1977) 0.04 (Cody, 1980)	93	1.8	21.3	0.8	StB	0.09 (Maberly et al., 1985)
Polyhalogenated biphenyls 															
3,5 (Cl)	PCB 14	AS[c]	34883-41-5	2	10	n.r.	–	NB	0.6 (Chauhan et al., 2000)	16	10	n.r.	–	SlB	–
2′,3,5 (Cl)	PCB 34		3768-68-5	8	10	n.r.	–	NB	0.3 (Chauhan et al., 2000)	<1	10	n.r.	–	NB	–
3,4,5 (Cl)	PCB 38		53555-66-1	11	10	n.r.	–	NB	1.9 (Chauhan et al., 2000) NB[d] (Purkey et al., 2004)	13	10	n.r.	–	NB	–
2,3′,5′,6 (Cl)	PCB 73		74338-23-1	3	10	n.r.	–	NB	–	<1	10	n.r.	–	NB	–
3,3′,5,5′ (Cl)	PCB 80		33284-52-5	<1	10	n.r.	–	NB	7.1 (Chauhan et al., 2000) 4.8 (Rickenbacher et al., 1986) Active[d] (Purkey et al., 2004)	4	10	n.r.	–	NB	–
2,2′,3,5′,6 (Cl)	PCB 95		38379-99-6	<1	10	n.r.	–	NB	0.5 (Chauhan et al., 2000)	3	10	n.r.	–	NB	–
2,3′,4,5′,6 (Cl)	PCB 121		56558-18-0	1	10	n.r.	–	NB	–	2	10	n.r.	–	NB	–
3,3′,4,5,5′ (Cl)	PCB 127		39635-33-1	<1	10	n.r.	–	NB	8.2 (Chauhan et al., 2000) Active[d] (Purkey et al., 2004)	9	10	n.r.	–	NB	–
2,2′,3,4,4′,5′ (Cl)	PCB 138		35065-28-2	5	10	n.r.	–	NB	1.8 (Chauhan et al., 2000) NB[d] (Purkey et al., 2004)	3	10	n.r.	–	NB	–
Hydroxy polyhalogenated biphenyls															
4-OH-2,2′,3,3′,4′, 5,5′6,6′ (F)	4-OH PFB	S-A	2894-87-3	95	10	11.2	1.4	VSB	–	95	10	66,050	2.6E−4	WB	
4-OH-3,5 (Cl)	4-OH PCB 14	AS	1137-59-3	98	0.2	16.9	1.1	VSB	8.5 (Rickenbacher et al., 1986) 3.9 (Cheek et al., 1993)	3	10	n.r.	–	NB	NB (Cheek et al., 1993)
3-OH-2′,3′,4′,5′ (Cl)	3-OH PCB 61		67651-37-0	8	10	n.r.	–	NB	1.9 (Cheek et al., 1993)	10	10	n.r.	–	NB	
4-OH-2′,3,4′,6′ (Cl)	4-OH PCB 69		189578-00-5	94	0.2	32.7	0.6	StB		<1	10	n.r.	–	NB	0.03 (Cheek et al., 1993)
4-OH-2′,3,5,5′ (Cl)	4-OH PCB 72		245084-57-5	97	0.2	7.3	2.5	VSB	–	88	10	1116	0.02	MB	
4-OH-2′,3,3′,4′,5′ (Cl)	4-OH PCB 106		192190-09-3	100	0.6	51.6	0.4	StB	0.4 (Cheek et al., 1993)	4	10	n.r.	–	NB	NB (Cheek et al., 1993)
4-OH-2′,3,4′,5,6′ (Cl)	4-OH PCB 121		190271-92-2	100	0.2	22.4	0.8	StB	1.6 (Cheek et al., 1993)	43	10	n.r.	–	SlB	NB (Cheek et al., 1993)
Polyhalogenated diphenyl ethers 															
2,2′,4,4′ (Br)	BDE 47	AS	5436-43-1	19	1	n.r.	–	SlB	$<2.2E^{-3}$ (Hamers et al., 2006) $2.5E^{-3}$ (Hamers et al., 2008)	24	10	n.r.	–	SlB	–
2,2′,4,5′ (Br)	BDE 49		243982-82-3	36	10	n.r.	–	SlB	$<2.2E^{-3}$ (Hamers et al., 2006)	67	16.2	2.5E3	0.01	WB	–
2,3′,4,5′ (Br)	BDE 68		446254-38-2	11	10	n.r.	–	NB	–	26	16.2	n.r.	–	SlB	–
2,2′,4,4′,5 (Br)	BDE 99		60348-60-9	24	10	n.r.	–	SlB	NB (Hamers et al., 2006)	71	16.2	5685	$3E^{-3}$	WB	–

Hydroxy polyhalogenated diphenyl ethers																
2-OH-2′,4,4′ (Cl)	Tricl	S-A	3380-34-5	98	10	669	0.03	MB	–	96	10	1182	0.02	MB	–
3-OH-2,2′,4,4′ (Br)	3-OH BDE 47	AS	24949-31-3	97	0.2	22.3	0.8	StB	4 (Hamers et al., 2008)	81	1.8	219	0.08	MB	–
5-OH-2,2′,4,4′ (Br)	5-OH BDE 47		602326-30-7	91	0.2	44.3	0.4	StB	3 (Hamers et al., 2008)	83	1.8	235	0.07	MB	–
6-OH-2,2′,4,4′ (Br)	6-OH BDE 47		79755-43-4	72	0.6	87	0.2	StB	0.3 (Hamers et al., 2006), 0.3 (Legler et al., 2002) 0.4 (Hamers et al., 2008)	65	0.6	110	0.16	StB	–
4′-OH-2,2′,4,5′ (Br)	4′-OH BDE 49		602326-23-8	69	0.2	107.8	0.2	StB	3.5 (Hamers et al., 2008)	79	1.8	867	0.02	MB	–
6′-OH-2,2′,4,5′ (Br)	6′-OH BDE 49	Pr[e]	497069-16-6	85	0.2	66	0.3	StB	–	65	0.6	191	0.09	MB	–
6-OH-2,2′,4,4′,5 (Br)	6-OH BDE 99	AS	297742-10-0	81	0.2	83.6	0.2	StB	–	72	0.2	100	0.18	StB	–
Methoxy polybrominated diphenyl ethers															
6-MeO-2,2′,4,4′	6-MeO BDE 47	Pr	102739-99-1	22	1.8	n.r.	–	SlB	–	33	10	n.r.	–	SlB	–
2′-MeO-2,3′,4,5′	2′-MeO BDE 68		96920-28-4	<1	1	n.r.	–	NB	–	17	10	n.r.	–	SlB	–
Diphenyl methanes															
															
Bisphenol A	BPA	S-A	80-05-7	18	10	n.r.	–	SlB	NB (Meerts et al., 2000)	12	10	n.r.	–	NB	–
Tetrachlorobisphenol A 2,2′,6,6′ (Cl)	TCBPA		79-95-8	87	0.2	32.4	0.6	StB	0.8 (Meerts et al., 2000)	26	10	n.r.	–	SlB	–
Tetrabromobisphenol A 2,2′,6,6′ (Br)	TBBPA		79-94-7	96	0.2	18.8	1	StB	10.6 (Meerts et al., 2000) 2.3 (Legler et al., 2002) 1.6 (Hamers et al., 2006)	5	10	n.r.	–	NB	–
Halogenated phenols															
															
2,4 difluorophenol	DFP	S-A	367-27-1	5	10	n.r.	–	NB	–	20	10	n.r.		SlB	–
2,6 difluorophenol	DFP		28177-48-2	2	10	n.r.	–	NB	–	3	10	n.r.		NB	–
2,4,6 trifluorophenol	TFP		2268-17-9	<1	10	n.r.	–	NB	–	6	10	n.r.	–	NB	–
2,4 dichlorophenol	DCP		120-83-2	19	10	n.r.	–	SlB	–	8	10	n.r.	–	NB	–
Pentachlorophenol	PCP		87-86-5	79	0.2	33.2	0.6	StB	2.5 (den Besten et al., 1991) 1.7 (van den Berg, 1990)	21	10	n.r.	–	SlB	0.001 (van den Berg, 1990)
2,4 dibromophenol	DBP		615-58-6	95	5.4	149.3	0.1	StB	0.06 (Meerts et al., 2000)	<1	10	n.r.	–	NB	–
2,4,6 tribromophenol	TBP		118-79-6	98	0.6	20.2	0.9	StB	1.2 (Meerts et al., 2000) 2 (Legler et al., 2002)	4	10	n.r.	–	NB	–
Pentabromophenol	PBP		608-71-9	88	0.2	26.6	0.7	StB	10.2 (Hamers et al., 2006)	21	10	n.r.	–	SlB	–

HTC. Highest tested concentration.
n.r. Not reached.
[a] The categories distinguished are, weak binders (WB, RP<0.01), moderate binders (MB, 0.01<RP<0.1), strong binders (StB, 0.1<RP<1) and very strong binders (VSB, RP>1). Non-binders (NB) and slight binders (SlB) inhibited the transport proteins less than 16% and 50% respectively.
[b] Sigma Aldrich.
[c] AccuStandard.
[d] RP value not available, assay based on immunoprecipitation-HPLC.
[e] Promochem.

Table 2
Data set of the 20 pharmaceuticals, pesticides and other compounds tested with the biosensor transport protein inhibition assays (categories are explained in Table 1, the structures of the compounds are presented in Fig. 3)

Compound	Abbreviation	Source	CAS	TTR						TBG					
				PI (%)	HTC (μM)	IC_{50} (nM)	RP	Category	Literature RP	PI (%)	HTC (μM)	IC_{50} (nM)	RP	Category	Literature RP
Pharmaceuticals (structures in Fig. 3A)															
Tetraiodothyroacetic acid	Tetrac	S-A[a]	67-30-1	96	10	17.2	1.1	VSB	11.5 (Rickenbacher et al., 1986) 8 (Somack et al., 1982) 0.8 (Cheng et al., 1977) 2.5 (Cody, 1980)	88	1.8	29	0.6	StB	0.4 (Hao and Tabachnick, 1971)
Ibuprofen	IBU		15687-27-1	31	10	n.r.	–	SlB	–	<1	10	n.r.	–	NB	–
Furosemide	Fur		54-31-9	15	10	n.r.	–	NB	NB (Munro et al., 1989)	1	10	n.r.	–	NB	$1.1E^{-3}$ (Munro et al., 1989)
Sodium diclofenac	Na Dic		15307-79-6	83	5.4	936	0.02	MB	0.02 (Munro et al., 1989)	75	5.4	1360	0.01	MB	$3.9E^{-4}$ (Munro et al., 1989)
Mefenamic acid	Me Ac		61-68-7	53	1.8	1.2E[c]	0.02	MB	0.3 (Munro et al., 1989)	15	10	n.r.		NB	$2.7E^{-4}$ (Munro et al., 1989)
Bezafibrate	BFB		41859-67-0	<1	10	n.r.	–	NB	–	<1	10	n.r.	–	NB	–
Clofibrate	CBF		637-07-0	<1	10	n.r.	–	NB	–	<1	10	n.r.	–	NB	–
Clofibric acid	CFA		882-09-7	<1	10	n.r.	–	NB	–	<1	10	n.r.	–	NB	–
Diatrizoic acid	DTA		117-96-4	<1	10	n.r.	–	NB	–	<1	10	n.r.	–	NB	–
Carbamazepine	CMZ		298-46-4	<1	10	n.r.	–	NB	–	<1	10	n.r.	–	NB	–
Pesticides and Others (structures in Fig. 3B)															
Genistein	Gen	S-A	446-72-0	91	10	43.8	0.4	StB	KD=40 nM (Green et al., 2005)	1	10	n.r.	–	NB	–
Dichlofluanid	DCF		1085-98-9	<1	10	n.r.	–	NB	–	<1	10	n.r.	–	NB	–
Trifluoroacetic acid	TFAA		76-05-1	<1	10	n.r.	–	NB	–	<1	10	n.r.	–	NB	–
Perfluorooctanesulfonate	PFO		111873-33-7	25	1	n.r.	–	SlB	–	<1	10	n.r.	–	NB	–
2-Fluoro-4-nitrophenol	2F-PNP		403-19-0	<1	10	n.r.	–	NB	–	<1	10	n.r.	–	NB	–
2-Nitro-4-(trifluoromethyl) phenol	NTFMP		400-99-7	15	1	n.r.	–	NB	–	<1	10	n.r.	–	NB	–
Trifluoperazine dihydrochloride	TFPDCl		440-17-5	<1	10	n.r.	–	NB	–	<1	10	n.r.	–	NB	–
l-Cyhalotrin	Cyhalo	AS[b]	91465-08-6	<1	10	n.r.	–	NB	–	<1	10	n.r.	–	NB	–
Chlorpyrifos	CPF	RdH[c]	2921-88-2	2	10	n.r.	–	NB	–	3	10	n.r.	–	NB	–
4-OctylPhenol	OP	Dr.E[d]	1806-26-4	26	10	n.r.	–	SlB	–	<1	10	n.r.		NB	–

HTC. Highest tested concentration.
[a] Sigma Aldrich.
[b] AccuStandard.
[c] Riedel-de Haën.
[d] Dr. Ehrenstorfer.

previously (Marchesini et al., 2006) with some modifications on the conditions to improve the transport protein stability and maximize the unattended operation time. Briefly, T4 was immobilized to the CM5 biosensor chip surface via the amino group of the alanyl side chain using AVA as spacer. The transport protein assays were performed in a Biacore Q that automatically mixed 20 μL of the transport protein solution (91 nM) with 80 μL of the analyte solution and immediately injected 50 μL of the mixture over the T4-coated sensor chip surface at a flow rate of 25 μL min^{-1}. The variation in response over time of each transport protein binding to the T4-coated sensor chip surface under different buffer conditions was used as a parameter of transport protein stability. The response, measured 15 s after the injection ended was used for data comparison and the surface was regenerated by injecting 15 μL of an acetonitrile (ACN) and 0.1 M NaOH (1:4; v/v) mixture.

In the final format, a phosphate buffer (NaH_2PO_4 (0.6 g), Na_3PO_4*$12H_2O$ (1.9 g), NaCl (0.59 g) per liter adjusted to pH 8.5) was used as running buffer. The transport protein stock solutions were prepared in running buffer with the addition of OVA (40 μg mL^{-1}) to preserve the transport protein stability. A typical maximum response of a blank buffer injection was 750 response units (RU). Stock solutions of the chemical standards (10 mM) were prepared in DMSO if possible, or otherwise in the organic solvent recommended by the provider. The T4 stock solution (10 mM) was prepared by dissolving T4 in a solution containing DMSO (94%), chloroform (5%) and 1 M NaOH (1%). The buffer used for dilution of the chemical standards was running buffer with the addition of 2% DMSO and ascorbic acid (0.5 g L^{-1}) as antioxidant. At least one T4 calibration curve was included in each set of analyte calibration standards.

Transport protein binding affinity. The binding affinity of a chemical to either rTTR or TBG was expressed as the relative potency (RP) of the compound compared to the natural hormone, T4. Therefore, concentrations inducing 50% inhibition (IC_{50}) were obtained from calibration curves of the compounds and T4. The RP is defined as the ratio between the IC_{50} of T4 and the IC_{50} of the compound [IC_{50} (T4)/IC_{50} (analyte)]. The mean IC_{50} of all the T4 calibration curves was used for the RP calculations. The mean IC_{50} of T4 in the TTR assay was 18.4±4.4 nM (n=15) and 17.2±1.2 nM (n=10) in the TBG assay. The RP of each analyte was calculated based on at least two full dose response curves based on six different concentrations. The chemicals were classified according to their percentages of inhibition at the highest tested concentrations. Active chemicals were those with an inhibition higher than 50% and were further classified based on their calculated RP. Namely designated as very strong binder (VSB) (RP>1), strong binder (STB) (1>RP>0.1), moderate binder (MB) (0.1>RP>0.01), or weak binder (WB) (RP<0.01). Those that did not reach an inhibition of 50% at the highest tested concentration were differentiated in two groups. Those chemicals inhibiting transport protein binding in the 0–15% range were designated as "non-binders" (NB) and those showing inhibition in the 16–49% range were designed as "slight binders" (SlB).

Results

Optimization of the assays

Typical sensorgrams obtained with the TBG assay for three different concentrations of T4 are shown in Fig. 1B. There is a relatively large bulk response in the sensorgrams caused by the presence of DMSO in the dilution buffer. After the injection finishes, the specific binding response is measured. The regeneration conditions effectively disrupted the transport protein-T4 interaction without damaging the T4-coated chip surface. The total run time for each cycle was 8 min, including mixing the transport protein stock solution with the sample and the washing steps. The T4-coated chip surface was very stable and could be used for over 1000 cycles. To allow long periods of unattended operation, it is critical that the T4 binding activity of the transport proteins remains stable. One of the factors affecting the stability of TTR in binding assays is its concentration in solution (Rickenbacher et al., 1986). TTR was exposed to different conditions to study the decrease in binding response to immobilized T4. When TTR was used as previously (Marchesini et al., 2006), the response obtained after the injection of (18.2 nM) TTR decreased 43% after 240 min (30 cycles). At this concentration, the TTR stability was greatly improved after the addition of (182 nM) OVA as a stabilizing agent and the response decreased only 12% after 240 min of serial injections. OVA has a reported low affinity for T3 and T4 (Korcek and Tabachnick, 1976) and is therefore interesting as stabilizing agent because it would have a minor effect on the relative affinities of the analytes to the transport proteins. The stability of TTR was further improved by preparing a five-fold more concentrated transport protein and OVA stock solution. Prior to each injection, the Biacore Q mixed the transport protein stock solution with the analyte solution (1/5; v/v). The measurement of a full set of 7 calibration curves (three analytes in duplicate and T4) took 960 min. Under these conditions, the transport protein stock solutions remained stable and showed minimum response decrease (<5% for rTTR and<2% for TBG). Additional improvements were the presence of 2% DMSO in the dilution buffer, which enhanced the solubility of hydrophobic chemicals allowing the screening of chemicals over a wider range of Log P values, and the presence of 2.8 mM ascorbic acid to prevent oxidation of less stable metabolites (van Lipzig et al., 2005).

Under the improved conditions, both biosensor transport protein-based assays were more robust than under the previously used conditions (Marchesini et al., 2006). The RP values obtained previously with the same stock solutions of 4-OH PCB14, TBBPA, TCBPA, PBP, PCP and T3 showed similar RP values under the optimized conditions. The IC_{50} values for T4 increased in the TTR assay from 13.7 to 18.4 nM, and from 8.6 to 17.2 nM in the TBG assay. However, as other chemicals had concomitant IC_{50} increases, the RP value was not significantly changed. The only exception was 4-OH PCB14, showing a significant IC_{50} increase from 3.1 nM to 16.9 nM, thus, an interaction of 4-OH PCB 14 with OVA cannot be excluded as the cause. Hence, 4-OH PCB14 was excluded from further analysis.

The TBG assay with improved conditions showed a RP increase for T3 from 0.6 to 0.8 compared to the previous conditions.

A library of 18 chemicals (T4, T3, 4-OH PCB 69, 4-OH PCB 106, 3-OH BDE 47, 5-OH BDE 47, 6-OH BDE 47, 4′-OH BDE 49, 6′-OH BDE 49, 6-OH BDE 99, TCBPA, TBBPA, DBP, TBP, PCP, PBP, Na Dic, Me Ac) was tested in the TTR assay under the old and new conditions and had similar RPs, with a regression equation $RP_{(New\ Conditions)} = 1.06\ RP_{(Old\ Conditions)} - 0.03$ ($R^2 = 0.86$). Hence, the addition of DMSO, OVA and ascorbic acid to the transport protein-based assays had minor effects on the RP values.

Screening of a 62 chemicals library

From the 62 chemicals tested with the two transport protein-based assays, 25 were classified as active for producing more than 50% inhibition of TTR and 15 of TBG (Fig. 2). Of the library, 28 compounds were non-binders (producing less than 16% inhibition) to TTR and 37 to TBG. The remaining 9 chemicals for TTR and 10 for TBG, were slight binders with more than 15% inhibition but less than 50%. The 25 compounds active towards TTR had RPs ranging from 0.02 up to 2.5. For the more specific TBG, 15 compounds were active, but none of them was more potent than T4 itself. The RPs of the TTR active chemicals mostly were centered around the STB category (17 chemicals) and around the MB category for TBG (7 compounds) (Fig. 2).

Detailed information about each chemical, i.e., the chemical name, structure, source, CAS number and measured binding affinity, as well as those found in literature (IC_{50} and RP) is presented in Tables 1 and 2. Structures of the compounds from Table 2 (pharmaceuticals, pesticides and other chemicals), are presented in Fig. 3.

The active hormone (T3) is also active for binding the pro-hormone (T4) transport proteins and had an RP compared to T4 of 0.04 in the TTR assay and 0.8 in the TBG assay (Table 1). All tested polychlorinated biphenyls (PCBs) were inactive in both biosensor transport protein-based assays. Even PCBs structurally resembling T4 (PCBs 73, 80, 121, 127), did not interact with TTR nor with TBG. The 5 para-hydroxylated PCBs (OH PCBs) were strong TTR binders (RPs ranging from 0.4 till 2.5). The highly substituted, hydroxylated polyfluorinated biphenyl (4-OH PFB) showed a very strong affinity for TTR (RP = 1.4). The general affinity ranking of this group of chemicals for TTR was 4-OH PCB72 > 4-OH PFB > 4-OH PCB 14 > 4-OH PCB121 > 4-OH PCB 69 > 4-OH PCB 106 > 3-OH PCB 61 (NB) (Table 2). The OH polyhalogenated biphenyls (PHBs) did not bind to TBG, except for the moderate binder 4-OH PCB 72 and the weak binder 4-OH PFB.

All polybrominated diphenyl ethers (PBDEs) tested were inactive in the TTR assay. In the TBG assay, BDE 49 and BDE 99 were weak binders (WB) with RPs of 0.01 and 0.003, respectively. However, all hydroxylated polyhalogenated diphenylethers (OH PHDEs) tested were moderate to strong binders to TTR. The most potent binder to TTR was 3-OH BDE 47 (RP of 0.8), the least potent was TricI (RP of 0.03). The affinity ranking for the OH-PBDEs for TTR was 3-OH BDE 47 > 5-OH BDE 47 > 6′-OH BDE 49 > 6-OH BDE 99 = 6-OH BDE 47 = 4′OH BDE 49 followed by TricI.

The OH PHDEs tested were also slight (6-OH BDE 47 and 6-OH BDE 99) to moderate binders (including TricI) in the TBG assay. The TBG affinity ranking was 6-OH BDE 99 > 6-OH BDE 47 > 6′-OH BDE 49 > 3-OH BDE 47 > 5-OH BDE 47 > 4′OH BDE 49 followed by TricI. From the two methoxy (MeO) PBDEs tested, 6-MeO BDE 47 had a slight affinity for both transport proteins and 2′-MeO BDE 68 only for TBG.

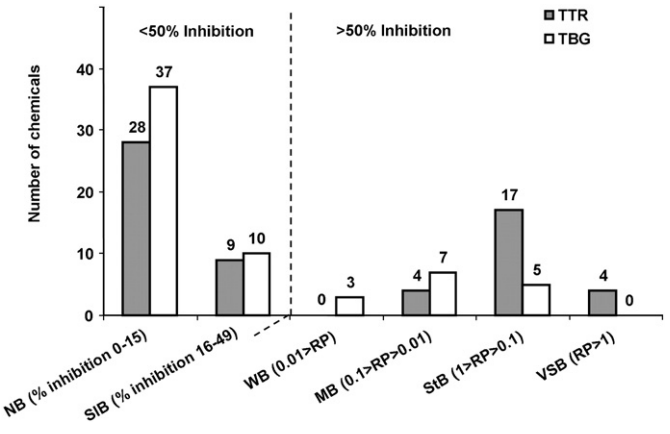


Fig. 2. Distribution of the RPs compared to T4 of the 62 tested chemicals determined with the TTR- and TBG inhibition assays. The compounds are classified based on their percentage of transport protein inhibition if they do not reach 50%, or based on their relative binding potency. The categories distinguished are, weak binders (WB, RP<0.01), moderate binders (MB, 0.01<RP<0.1), strong binders (StB, 0.1<RP<1) and very strong binders (VSB, RP>1). Non-binders (NB) and slight binders (SlB) inhibited the transport protein's less than 16% and 50% respectively.

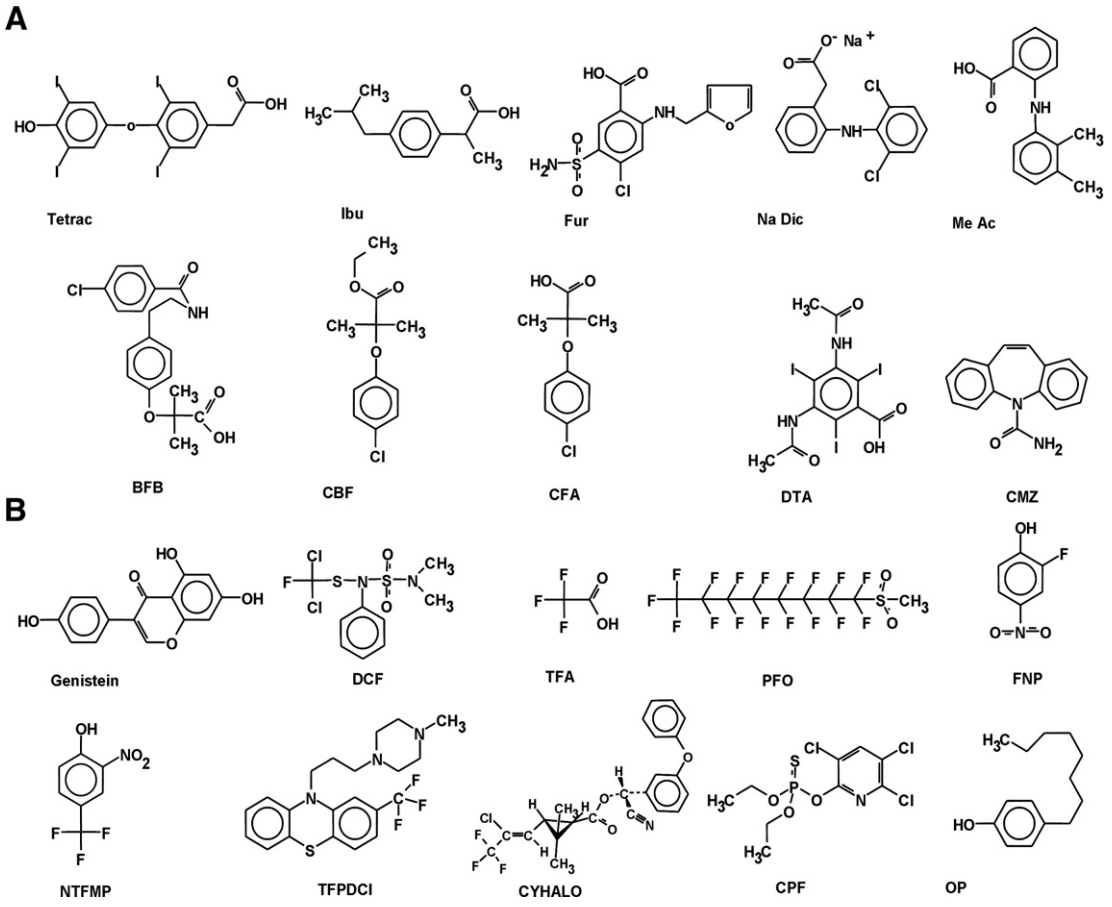


Fig. 3. Structures of pharmaceuticals (A) and pesticides and other chemicals (B) presented in Table 2 and tested in the transport protein-based assays.

Although the unsubstituted diphenyl methane (BPA) tested in the transport protein-based assays was inactive, the halogenated congeners were strong binders in the TTR assay with RP values of 0.6 for TCBPA and 1 for TBBPA, but both were inactive in the TBG assay.

None of the fluorinated phenols was active in the transport protein-based assays, of the chlorinated phenols only pentachlorophenol had affinity for binding TTR (RP=0.6) (Table 1). Interestingly, all the brominated phenols were active TTR binders with RPs up to 0.9 for tribromophenol (TBP).

In the TBG assay only DFP, PCP and PBP were slight binders, but showing less than 20% inhibition.

Three of the ten common pharmaceuticals tested (Fig. 3A) were active in the TTR assay (Table 2), with the T4 like Tetrac being the most potent (RP of 1.1). Sodium diclofenac (Na Dic) and mefenamic acid (Me Ac) were less potent (RPs of 0.02). In the TBG assay only Tetrac (RP of 0.6) and NaDic (RP=0.01) were active.

Of the 10 pesticides and other compounds of environmental relevance tested (Fig. 3B), only the natural isoflavonoid, genistein (Gen), was active in the TTR assay. All these chemicals were inactive in the TBG assay.

To compare the affinity of TTR and TBG of the chemicals tested, the RP of the active chemicals for TTR was plotted versus the RP of the chemical for TBG in Fig. 4. Those chemicals that inhibited the transport protein-based assay less 50% were plotted using the $-\mathrm{Log}_{10}$ of the percentage of inhibition (PI%) at the highest concentration tested. Inactive compounds showing a PI% for either of the transport proteins below 1 were assigned a Log_{10} value of 0 and are therefore aligned with the axis. The shaded quadrant of the plot contains a group of 13 chemicals that were both active for TTR and TBG. Most of these compounds are located below the T4 diagonal and along the TTR axis showing in general a higher RP for TTR than for TBG. Four active compounds (TriCl, 6-OH BDE 47, 6-OH BDE 99) are aligned with the T4 diagonal indicating similar relative affinity for both TTR and TBG. The only compound that was more potent for TBG than for TTR, was T3, located above the diagonal. The following quadrant clockwise depicts 12 compounds active only in the TTR assay, one of them, 4-OH PCB14, being a very strong binder. A subgroup of four compounds (PCP, PBP, TClBPA and PCB121) all highly substituted and strong binders for TTR are located in the SlB strip for TBG.

The next quadrant clockwise contains 36 compounds showing less than 50% inhibition in both transport proteins, 24 were non-binders for both TBG and TTR, 10 were slight binders with one of the transport proteins. Interestingly, 2 compounds (6-Me BDE47 and BDE47) were slight binders with both transport proteins and were aligned with the T4 diagonal. The last quadrant is virtually empty except for two compounds (BDE49 and BDE99) that had a much higher relative affinity for TBG than for TTR.

Discussion

In our previous study we characterized TTR and TBG using a SPR-based biosensor and this allowed us to develop two T4 transport protein inhibition assays using this system (Marchesini et al., 2006). In the present study these assays were successfully optimized to endure the conditions needed for screening a large library of 62 chemicals. We discovered that several hydroxylated metabolites of commonly used brominated flame retardants have an unexpectedly strong affinity for TBG. All of these compounds also have a strong affinity for binding TTR.

When our results are compared with those reported in literature (Tables 1 and 2), the first issue that arises is the difference in the RPs obtained with those previously reported. One fundamental reason to

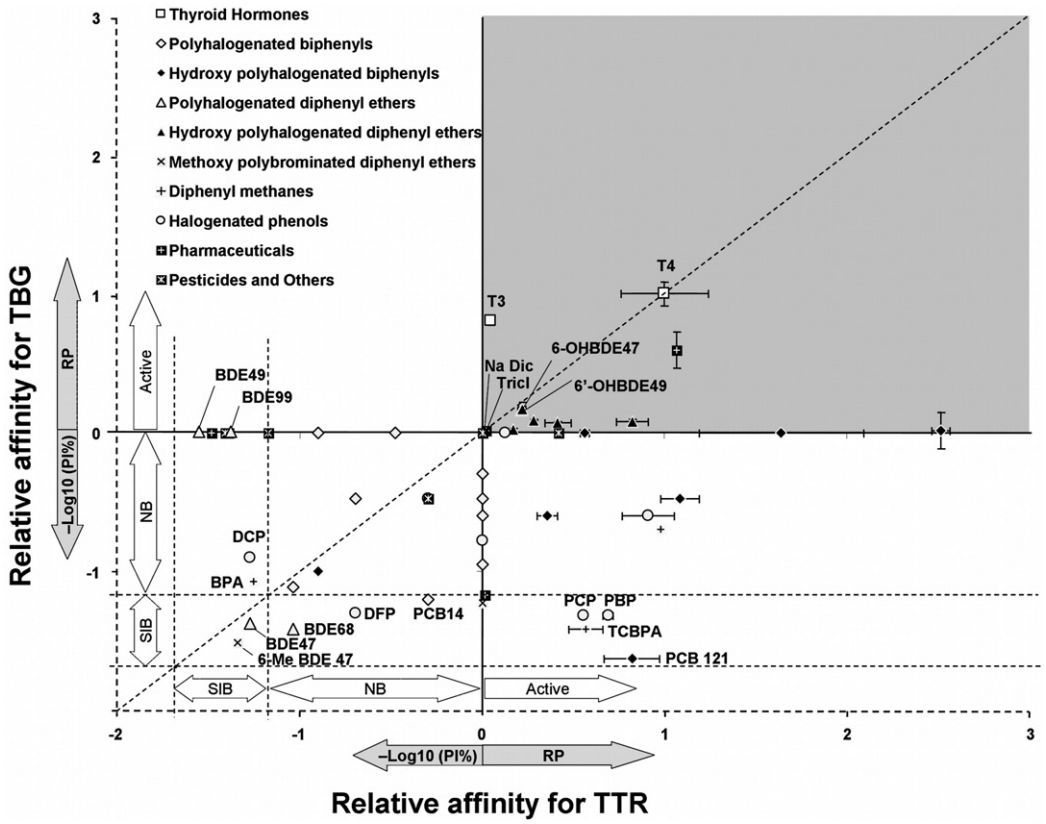


Fig. 4. The affinity of the 62 chemicals tested for TTR and TBG binding. The $-\text{Log}_{10}$ of the percentage of inhibition (PI%) was used to plot compounds not reaching 50% inhibition. Compounds for which the relative potency (RP) compared to T4 was calculated, were plotted based on their RP. Those compounds in the shaded quadrant have a strong affinity for both TTR and TBG.

explain these differences is the TTR source. The classical T4 radioligand binding assay (RBA) uses TTR purified from human serum that is probably less stable than the recombinant TTR used in this study. Additionally, TTR has two allosteric binding sites, a low and a high affinity one and the structure of the ligands plays a crucial role in the TTR binding kinetics (McCammon et al., 2002). Potentially generating complex binding kinetics when the isotope-labeled T4 is bound to one binding site, thus influencing the binding kinetics of the other ligand to the second binding site, or vice-versa. Another important difference between our TTR assay and the TTR RBA is that the latter is performed in a pure aqueous solution, whereas our assay includes ascorbic acid, 2% DMSO and OVA creating more stable conditions for lipophilic compounds.

Furthermore, it has been reported that even in similar experiments using TTR radioligand binding assays, the RP values for a single compound may differ up to 6 fold (i.e. TBBPA) (Meerts et al., 2000; Hamers et al., 2006). The RP variations reported confirm previous reports indicating that TTR is very sensitive to slight changes in assay conditions (Chauhan et al., 2000; Marchesini et al., 2006). The dissimilar methods and assay protocols used complicate quantitative inter method or inter laboratory comparisons. However, qualitatively most of the RP values from different studies, including ours, are in accordance with each other.

The RP of T3 (0.04) in our study was comparable with the average RP values (0.06 ± 0.03) reported in literature for TTR. The RP for T3 (0.8) in the TBG assay was ten times higher than previously obtained (Maberly et al., 1985). Korcek and Tabachnick show that the affinity of TBG for T3 is relatively higher than T4 at a higher pH (Korcek and Tabachnick, 1976). Hence, using a pH of 8.5 for the TBG assay in the present study probably increased the RP for T3.

In our transport protein-based assays all the PCBs tested were inactive. Contrasting with the results of Chauhan et al., who reported that several of these compounds interacted strongly with TTR using a RBA (Chauhan et al., 2000). Later, Purkey et al. could only confirm an interaction of only two out of the eight PCBs used by Chauhan (Purkey et al., 2004). In general, most of the previous studies show that parent PCBs were inactive for binding TTR. On the contrary, their meta or para-hydroxylated metabolites with one or more adjacent halogens substituents were potent T4 analogues (Rickenbacher et al., 1986; Lans et al., 1993). Our results with the TTR assay confirmed the TTR binding affinity for OH PCBs and discovered that 4-OH PFB was a very strong TTR binder.

Of the 4 PBDEs tested, 2 were weak binders, inhibiting TBG more than 50% and 3 were slight binders to TTR, inhibiting less than 50%. However, the OH-PBDE metabolites were all active and strong binders in both transport proteins. For TTR, the 3 meta OH group with two adjacent halogens, as present in 3-OH BDE 47, provided the optimum structure for binding to TTR, in accordance with previous results with OH PCBs (Rickenbacher et al., 1986; Lans et al., 1993). In 5-OH BDE 47 the presence of a OH group in the 5 meta position adjacent to one halogen reduced the RP 2-fold. The RP decreased a further 2-fold with an ortho OH group without adjacent halogens (as in 6-OH BDE 47). This decreasing trend in RP is in accordance with literature (see Table 1). One exception is 4-OH BDE49 that had a 2-fold lower RP than 5-OH BDE47 and one order of magnitude lower in the literature.

Our study is the first to show that environmentally relevant compounds can bind to TBG, complementing numerous reports about compounds binding to TTR. A different structure-related trend for TBG is observed if compared with TTR. The presence of a OH group in the ortho position without an adjacent bromine group seems to favor the binding to TBG, as seen for 6-OH BDE 47 and 6-OH BDE 99. However, TriCl also has an OH group in the ortho position and lacks adjacent chlorines, but shows the lowest RP of the group. This suggests a strong

effect of the halogen substituent size for binding TBG and explaining the lack of TBG binding to PCBs (Lans et al., 1994). The two strongest TTR binders in the group of the OH PHBs were also active in the TBG assay and had the same affinity ranking as for TTR. The inactivity of 4-OH PCB 106, 4-OH PCB 121 and 4-OH PCB14 for binding TBG was confirmed (Table 1).

All the compounds that were slight binders for TBG were at least tetra-substituted as shown in the second quadrant of Fig. 4 indicating the importance of the number of halogens in the ligand. One bromine adjacent to the OH group reduces the RP by about 2-fold, as seen with 6-OH BDE 49. This reduction is also seen with one or two bromines adjacent to the meta OH group with 3-OH BDE 47 and 5-OH BDE 47. The abundant presence of OH-PBDEs in the in vivo situation and medium to strong binders of TTR and TBG highlights the importance of this group of metabolites. PBDEs are easily hydroxylated and are persistent in blood, contributing to the PBDE mediated toxicity (Hakk and Letcher, 2003; Costa and Giordano, 2007). Of special interest are the OH metabolites of BDE 47 because they are the most common ones in humans (Letcher et al., 2004). Comparable RPs were shown by 6-OH BDE 47 for both transport proteins suggesting that high levels of this metabolite in human blood is a potential health concern. Specially for a developing fetus because OH-PHAHs can be transported through the placenta if bound to TTR (Morse et al., 1996; Ulbrich and Stahlmann, 2004).

Triclosan (Tricl) is a hydroxylated polychlorinated diphenyl ether, strongly resembling T4 and targeted equally both TTR and TBG. Both, humans and the environment are exposed to Tricl because it is intensively used as antibacterial agent for example in dishwashing liquid, soap, toothpastes, mouthwashes, etc (Singer et al., 2002; Gomez et al., 2007). This continued exposure and strong transport protein binding affinities make Tricl an important candidate for future studies.

BPA is a widely used weak estrogenic contaminant that hardly inhibited transport protein binding. However, BPA can be transformed into compounds with thyroid hormone disrupting activity like tetra chlorinated BPA (TCBPA) during aqueous chlorination in sewage treatment or in contaminated drinking water hoses (Yamauchi et al., 2003; Gallard et al., 2004). Both TCBPA (0.6) and the widely used flame retardant tetrabrominated BPA (TBBPA) (1), were strong TTR binders in our studies.

The RPs we observed for the halogenated phenols in the TTR assay are in accordance with an interesting trend previously reported by Meerts et al. (2000). Given equal structures, the affinity increase with increasing atomic weight of the halogens substituents, for instance, TCBPA<TBBPA, DFP<DCP<DBP, TFP<TBP, PCP<PBP. According to these results, exclusively fluorine-substituted phenolic emerging chemicals are unlikely have an effect as thyroxine transport disruptors.

The transport protein assays were also used to test environmentally relevant pharmaceuticals (for review see Halling-Sorensen et al., 1998; Jones et al., 2001) having T4 activity (Koizumi et al., 1984; Hambsch et al., 1986). In accordance with previous studies Tetrac had a high RP for TTR in the range from 0.8 to 11.5 and Na Dic and Me Ac were moderate binders to TTR. Previous studies reported an interaction of Furosemide (Fur), Na Dic and Me Ac with TBG, but in our study Fur and Me Ac were only slight binders due to the lower maximum concentration tested compared to the one used in literature (Table 1).

Of the pesticides and other compounds of environmental relevance tested, only the natural isoflavonoid, genistein (Gen) was active in the TTR assay with a IC_{50} of 43.8 nM. Roughly comparable with the equilibrium dissociation constant of 40 nM previously reported (Green et al., 2005). Gen also has a weak estrogenic activity and is considered a beneficial nutraceutical for menopausal women. On the other hand, the health implications of the strong TTR binding potency still is unknown and of interest for future research.

Toxicological relevance of T4 transport protein binding

The T4 binding site of TBG has recently been solved and is located on the surface of TBG (Zhou et al., 2006). Apparently, TBG is an active T4 carrier specifically adapted to allow triggered and modulated release of T4 to specific tissues including the placenta (Robbins, 2000; Zhou et al., 2006). Given that the fetus initially has no iodine reserve, it is dependent on the iodine transferred by the mother. Consequently, there is an increased concentration of T4-TBG in the mother during pregnancy in order to supply an avid placental uptake (Andrea et al., 1980; Schussler, 2000). Therefore, one could speculate that during ontogenesis the displacement of the T4-TBG complex by the OH-PBDEs commonly found in humans, could have consequences in fetal development. In addition, TTR mediates the maternal to fetal T4-transport through the placenta and the delivery of T4 across the blood-brain barrier. Previous studies show that compounds bound to TTR are transported to the fetal compartment and fetal brain, decreasing fetal brain T4 levels (Elst et al., 1998; Meerts et al., 2002).

The OH-PBDEs were the most active compounds toward both T4 transport proteins, however, these compounds usually occur in mixtures in combination with the OH PCBs which are more potent toward TTR. This study is the first to show that some OH-PHAHs have a strong binding affinity for TBG and one important requirement for binding is the presence of the bulkier bromine substituents found in OH-PBDEs. In addition to transport protein binding, T4 and T3-like OH-PBDEs also have been shown to induce T3-dependent cell proliferation in the T-screen and activate the nuclear TH receptors TRα and TRβ in specific reporter gene assays (Schriks et al., 2007). Moreover, OH-PHAHs can interfere with sulfonostransferasas (Schuur et al., 1998a; 1998b) and deiodinases (Adams et al., 1990). The total OH-PBDEs concentration in human blood is in the range of 0.01–0.5 nM and an order of magnitude higher for the PBDEs (Sjodin et al., 2004, Athanasiadou et al., 2008). Comparing these concentration with the 10–20 pM level of free T4 and 60–160 nM total T4 found in human blood (Schell et al., 2008) and the apparent accumulation of HO-PBDEs (Athanasiadou et al., 2008) raises serious concerns about disruption of T4 transport to the fetus. The OH metabolites of PHAHs (PCBs and PBDEs) occurring usually in mixtures may hamper the TH homeostasis at different physiological levels and via multiple thyroid hormone-related mechanisms (Schell et al., 2008) evidence the need for future research.

The fast and reproducible optimized SPR biosensor-based transport protein assay presented offers a suitable method for high throughput screening of a large library of compounds, their metabolites and even biological samples. As the T4 is immobilized on the chip surface in this method, it could be further expanded to measure binding of compounds to T4 sulfono- and glucuronyl-transferases and T4-iodinases. We speculate that the T4 binding sites of some of these enzymes may share features with those of the transport proteins, allowing their binding to the immobilized T4. For example, Tricl has been shown to interact strongly with both TBG and TTR in the present study and previous research reported a strong interaction with sulfonotransferases and glucuronyl-transferases (Wang et al., 2004), suggesting a possible predictive value of the transport protein-based assays for other binding assays. The combination of T4- and T3-assays currently under study with the latest SPR technology with multiple biosensing spots (Steiner, 2004; Chinowsky et al., 2007) would allow a drastic reduction in the number of tests needed for the screening of TH-disrupting potencies of compounds as required for EU regulations of toxic compounds such as REACH (EEC, 2006). Future studies should attempt to assess endocrine disrupting chemicals in a more comprehensive approach. Multi-system assays or multi-level assays could be developed not only for screening chemical standards but also the total bioactive toxicity load of real samples.

Acknowledgments

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