

Probing the Binding of BFRs to Thyroid Transport Proteins using NMR and Molecular Modeling

Darcy C. Burns¹; Kristian T. Levey¹; Gord C. Balch¹; Mehran Alaei², Chris D. Metcalfe¹

¹Worsfold Water Quality Centre, Trent University, Peterborough ON, K9J 7B8, Canada

²National Water Research Institute, Environment Canada, P.O. Box 5050 Burlington ON, L7R 4A6, Canada

Introduction. There is evidence that brominated flame retardants (BFRs) may disrupt the thyroid endocrine system of vertebrates by competitively inhibiting thyroid hormone binding to transport proteins such as human transthyretin (hTTR) (Balch et al. 2006). For instance, *in vitro* studies (Meerts et al. 2000) found that an unidentified metabolite(s) of BDE47 generated by CYP2B-enriched liver microsomes was capable of competitively inhibiting >60% of thyroxine (T4) from binding to human transthyretin (hTTR). Meerts et al. (2000) also found that the inhibitory effect of BDE99 on T4 binding was lower, at 20-60% competitive inhibition.

NMR spectroscopy combined with molecular modeling together comprise a unique approach for investigating protein-ligand interactions and thus are ideally suited to probe the capacity of BFRs to competitively bind thyroid hormone transport proteins. We have applied 1D ¹H and 1D ¹H saturation transfer difference (STD) NMR spectroscopy (Mayer and Meyer 1999) to study binding interactions between bisphenol A (BPA) and hTTR and inhibition of the binding of BPA to TTR by L-thyroxine (T4), 4,4'-Isopropylidenebis(2,6-dibromophenol) (TBBPA), 2,2',4,4'-Tetrabromodiphenyl ether (PBDE47), and 3-Hydroxy-2,2',4,4'-Tetrabromodiphenyl ether (3-hydroxy-PBDE47). Automated docking (Morris et al. 1996, Morris et al. 1998, Goodsell and Olsen 1990) was performed to assess the nature of binding between hTTR and T4, BPA, TBBPA, PBDE47, and 3-Hydroxy-PBDE47. These techniques can be used as a general tool to study the action of emerging contaminants, specifically BFRs and their metabolites, on thyroid regulation in vertebrate species.

Materials and Methods. hTTR, BPA, TBBPA, D₂O, and d₆-DMSO were purchased from Sigma Aldrich (St. Louis, MO), L-thyroxine was purchased from Acros Organics (Geel, Belgium), PBDE47 was purchased from Chem Service (West Chester, PA), and 3-Hydroxy-PBDE47 was purchased from AccuStandard (New Haven, CT). All chemicals were used as purchased without further purification. NMR experiments were performed at 25°C on a 500 MHz Varian Unity INOVA spectrometer equipped with a 5mm PFG triple-resonance indirect detection probe. The NMR samples were prepared by dissolving each of the ligands (T4, TBBPA, PBDE47, 3-hydroxy-PBDE47) with varying amounts of hTTR (0.0 mM, 5.0 mM) and bisphenol A (0.25 mM, 0.5 mM) in 90% phosphate buffer (0.02 M, pH 7.34) and 10% d₆-DMSO. Each NMR sample was supplemented with a capillary tube containing TSP dissolved in D₂O (0.75 % v.v.) for external referencing. 1D ¹H water-suppression experiments were carried out using a DPFGSE (double pulsed field spin gradient echo) suppression scheme with 128 transients, a 1.6 s delay, and 16384 points over a 5971 Hz spectral window. Spectra were baseline corrected and externally referenced to TSP (0.0 ppm). 1D ¹H saturation transfer difference (STD) experiments were carried out using a DPFGSE suppression scheme with 2048 transients, a 1.6 s delay, and

16384 points over a 5971 Hz spectral window. Selective protein saturation was achieved using a train of 40 Gaussian pulses of 52.3 ms duration, while residual protein signals were suppressed using a 20 ms spinlock filter. Baseline correction and 5 Hz line broadening were applied during processing and the spectra were externally referenced to TSP (0.0 ppm). STD_i and STD_o were determined as the maximum S/N achieved over a 200 Hz window.

Molecular modeling required the lowest-energy conformers of BPA, TBBPA, T4, PBDE47, and 3-Hydroxy-PBDE47 to be imported as pdb ligand files for use in AutoDock 3.0. The ground state equilibrium geometries and partial electrostatic charges of these ligands were calculated, *a priori*, using a semi-empirical PM3 approach (Spartan Pro V1.0.1; Wavefunction Inc., Irvine, CA). The crystal structure of hTTR (Wojtczak et al. 2001) was imported to AutoDockTools (a GUI for AutoDock 3.0), where water and thyroxine groups were subsequently removed. Polar hydrogens, united-atom Kollman charges, and atomic solvation parameters were added to the hTTR pdb model and automated docking was performed using a grid map of 80x80x80 grid points³ with a spacing of 0.375 Å centred in either binding site I or site II of hTTR. A total of 100 Lamarckian Genetic algorithm energy minimizations were performed with the following parameters: a population size of 250 individuals; 2.5×10^6 energy calculations; 27000 generations. In addition, 50 local search minimizations were performed with a maximum of 3000 energy calculations per minimization. All other parameters were set to their default values.

Results and Discussion. The ¹H STD spectrum of BPA in the presence of hTTR yielded peaks corresponding to the BPA phenyl ring protons (6.8 ppm, and 7.2 ppm) and methyl protons (1.6 ppm), indicating that protein binding was occurring (figure 1b). The phenyl ring proton S/N ratios were greater than that of the methyl protons, which suggests that BPA is oriented with the phenyl rings buried in the hTTR binding pocket and that these phenyl rings mediate hTTR binding. Molecular models of the lowest-energy docked complex between BPA and hTTR revealed BPA conformations that saddle the binding site, with each of the phenyl rings in close proximity to the walls of the hydrophobic P2 pocket.

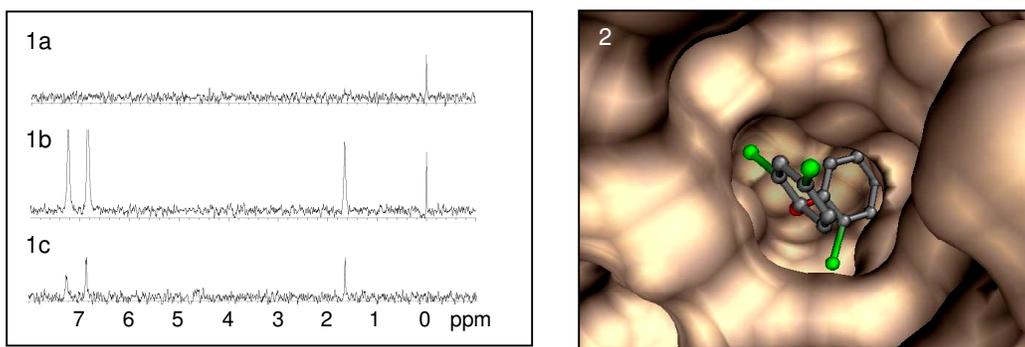


figure 1 ¹H 1D STD spectra for BPA binding to hTTR. a) 0.25 mM BPA, 0.0 μM hTTR, b) 0.25 mM BPA, 5.0 μM hTTR, c) 5.0 μM 3-Hydroxy-PBDE47, 5.0 mM BPA, 5.0 μM hTTR.

figure 2 ball and stick representation of the lowest-energy 3-Hydroxy-PBDE47 conformer bound to hTTR.

The addition of inhibitors to the BPA:hTTR ¹H STD NMR samples caused BPA peak integrations to become attenuated (figure 1c, table 1). Inhibition constants for T4, TBBPA, and the BFRs were calculated from these ¹H STD spectra using the procedure outlined by Wang et al (2004) and their inhibition potencies ranked as follows: 3-Hydroxy-PBDE47 > TBBPA > PBDE47 ~ T4 (table 1).

Molecular models of the lowest-energy inhibitor:hTTR complexes suggest that bromine atoms contribute to the tight binding of the BFRs in the P2/P3 pockets of hTTR. For PBDE47, the phenyl rings occupy the P2 and P3 binding pockets respectively. For T4 and 3-Hydroxy-PBDE47, binding occurs exclusively in forward mode with the hydroxyl group buried deep into the innermost P3 binding pocket (figure 2). Thus, the tight binding observed for the BFRs in the ^1H STD NMR spectra may arise from the combined influences of the phenyl ring, hydroxyl, and bromine moieties, which promote high affinity interactions in the deepest portion of the hTTR binding pockets.

table 1 Inhibition of BPA binding hTTR by thyroxine, TBBPA, PBDE47, and 3-Hydroxy-PBDE47, as determined from ^1H STD NMR. $\text{STD}_i/\text{STD}_o$ is the ratio of BPA phenyl ring S/N in the presence and absence of inhibitor. The binding affinities of some of these ligands have previously been reported and are listed here.

inhibitor	BPA S/N (200 Hz)	$\frac{\text{STD}_i}{\text{STD}_o}$	K_i ($\times 10^{-9}$ M) (experimental)	K_d ($\times 10^{-9}$ M) (literature)
-	25.9	-	-	1053 ^a
thyroxine	22.7	0.87	391.6	28.5 ^b
TBBPA	11.1	0.43	19.9	2.6 ^b
PBDE47	20.3	0.79	178.3	-
PBDE47OH3 ^c	9.6	0.37	3.24	-

^a Yamauchi et al. 2003. ^b Meerts et al. 2001. ^c ^1H NMR and ^1H STD NMR were acquired using a 3 mm Shigemi tube and referenced internally to d_6 -DMSO.

Acknowledgement. We wish to acknowledge the Natural Science and Engineering Research Council of Canada (NSERC) and Environment Canada for financial support.

References.

- Balch GC, Vélez-Espino LA, Sweet C, Alaei M, Metcalfe CD. 2006. *Chemosphere* 64:328-338.
- Goodsell DS, Olson AJ. 1990. *Proteins* 8: 195-202.
- Mayer M, Meyer B. 1999. *Angew Chem Int Ed* 38:1784-1788.
- Meerts IAT, van Zanden JJ, Luijckx EAC, van Leeuwen-Bol I, Marsh G, Jakobsson E, Bergman Å, Brouwer A. 2000. *Toxicol. Sci.* 56:95-104.
- Morris GM, Goodsell DS, Huey R, Olson AJ. 1996. *J Comput Aided Mol Des* 10:293-304.
- Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, Olson AJ. 1998. *J Comp Chem* 19:1639-1662.
- Wang Y, Liu D, Wyss DF. 2004. *Magn Reson Chem* 42:485-489.
- Wojtczak A, Neumann P, Cody V. 2001. *Acta Crystallogr Sect D* 57:957-967.
- Yamauchi K, Ishihara A, Fukazawa H, Terao Y. 2003. *Toxicol Appl Pharmacol* 187:110-117.