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Binding of alkylphenols and alkylated non-phenolics to the rainbow trout (*Oncorhynchus mykiss*) plasma sex steroid-binding protein

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Abstract

Alkylphenols are well-known endocrine disrupters, mediating effects through the estrogen receptor (ER). Although the estrogenic properties of the alkylphenols are well documented, alternative mechanisms of action are poorly described. In the present work, the interaction of a range of alkyl-substituted phenols and alkyl-substituted non-phenolics with the rainbow trout (*Oncorhynchus mykiss*) sex steroid-binding protein (rtSBP) were determined by competitive ligand-binding studies. The role of alkyl chain length and branching, substituent position, number of alkylated groups, and the requirement of a phenolic ring structure were assessed. The results showed that the rtSBP binds to most chemical structures tested, although the highest affinity was obtained for mono-substituted alkylphenols with a chain length of four to eight methyl groups. Interestingly, rtSBP binding was also observed for non-phenolic compounds such as 4-*t*-butylcyclohexanol and 4-*t*-butylnitrobenzene suggesting that the rtSBP has a broad binding specificity for alkylphenols and alkylated non-phenolics.

Keywords: Sex hormone-binding globulin; SHBG; SBP; Endocrine disrupter; Xenoestrogens; Nonylphenol; Octylphenol; Butylphenol; Structure–activity relationship; Fish

1. Introduction

Alkylphenols are a group of chemicals used in various industrial and household products such as detergents, platsticizers, and UV stabilizers and have been found as ubiquitous pollutants in waters worldwide. In recent decades, focus has been placed on alkylphenols due to their ability to cause feminization and inhibition of testicular growth in aquatic vertebrates such as fish (Jobling et al., 1996; Sumpter, 1995). These endocrine disruptors have been documented to bind to the fish estrogen receptor (ER), stimulate estrogen-mediated gene expression as well as hepatic synthesis of the egg yolk protein precursor vitellogenin and the eggshell (*zona radiata*) proteins in vitro and in vivo (Arukwe et al., 1997b; Celius et al., 1999; Flouriot et al., 1995; Knudsen and Pottinger, 1999; Olsen et al., 2005; Pelissero et al., 1993; Yadetie et al., 1999; Tollefsen et al., 2002a). Some alkylphenol isomers have also been documented to interfere with ER regulation (Knudsen et al., 1998) and steroid metabolism (Arukwe et al., 1997a). Recent studies have also reported that the alkylphenol 4-*t*-octylphenol was able to bind to specific sex steroid-binding proteins (SBPs) in plasma of fish and potentially modulate the endocrine system (Tollefsen, 2002; Tollefsen et al., 2004).

The plasma sex SBP, which is produced in the liver of fish (Foucher et al., 1991) and sequestered into the blood stream, binds sex steroids such as 17β-estradiol (E2) and testosterone (T) with high affinity and moderate capacity (Tollefsen, 2002; Øvrevik et al., 2001). As much as 95–98% of total sex steroids are bound to SBPs and low-affinity binding proteins such as albumin and corticosteroid-binding proteins (Westphal, 1986). Although the complete physiological role of SBPs is not currently known, it is proposed that this protein regulates the transport, cellular uptake, excretion and bioavailability of steroids as well as being involved in intracellular signaling to nuclear

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receptors (Fortunati, 1999; Petra et al., 1985; Rosner, 1990). Interestingly, a large number of environmental estrogens are able to interfere with steroid binding to fish SBPs in vitro (Kloas et al., 2000; Milligan et al., 1998; Tollefsen, 2002; Tollefsen et al., 2004) and modulate the levels of circulating SBP in vivo (Tollefsen et al., 2002b). Recent studies have shown that similar in vitro and in vivo effects can be induced by exposure to complex synthetic mixtures and industrial effluents (Tollefsen et al., 2006; Tollefsen, pers. commun.).

Although several reports document the estrogenic potential of alkylphenols, few studies have determined the potential of alkylphenols to interfere with the natural functions of the SBP. In the present work, a mechanistically oriented structure–activity relationship approach has been adopted to determine the structural binding requirements of the plasma SBP from rainbow trout (*Oncorhynchus mykiss*) for a range of alkylphenols and alkylated non-phenolics. The ligand-binding specificity of the SBP was determined by assessing the role of alkyl chain size and form (branching), the position and number of alkylated groups as well as the requirement of a phenolic ring structure on the binding affinity for the rainbow trout SBP (rtSBP).

2. Materials and methods

2.1. Chemicals

The test chemicals 17β-estradiol (E2), phenol, 2-*t*-butylphenol, 3-*t*-butylphenol, 2,4-di-*t*-butylphenol, 2,4,6-tri-*t*-butylphenol, 4-*n*-octylphenol and 4-*t*-octylphenol were all from Sigma-Aldrich (St. Louis, MI, USA). 4-methylphenol, 4-ethylphenol, 4-*n*-propylphenol, 4-*n*-butylphenol, 4-*s*-butylphenol, 4-*t*-pentylphenol, 4-*n*-hexylphenol and 4-*t*-butyltoluene were from TCI (Tokyo, Japan). 4-*t*-butylphenol, 4-*n*-nonylphenol, *cis*/*trans* 4-*t*-butylcyclohexanol, 4-*t*-butylbenzene, 4-*t*-butylbenzoic acid, 4-*t*-butylbenzaldehyde, 4-*n*-chlorobenzene, 4-*t*-butylbromobenzene, 4-*t*-butyliodobenzene, 4-*t*-butylnitrobenzene, 4-*n*-butylaniline and 4-*t*-butylthiophenol were from Lancaster (Morecamble, UK). 4-*n*-pentylphenol and an isomeric mixture of 4-dodecylphenol were obtained from Acros (Geel, Belgium). 4-*n*-heptylphenol was supplied by Eastman Kodak (Rochester, NY, USA). 1,2,4,6,7-[^{3}H]-estradiol, [^{3}H]-E2 (85 Ci/mmol) were bought from Amersham plc. (Buckinghamshire, England). All the test chemicals had a purity of minimum 96%. Prior to use all chemicals were diluted in methanol (99.9%) and stored at −80 °C.

2.2. Fish

Rainbow trout, obtained from Killi Oppdrettsanlegg (Dombås, Norway) were kept in tanks at the Institute of Biology, University of Oslo (Norway) at a water temperature of 12 °C, oxygen saturation of approximately 100% and a pH of 6.6. The fish were fed daily with commercial fish pellets (EWOS, Bergen, Norway) in amounts corresponding to 0.5% of total body mass. The tanks received artificial illumination (100 lux) for 12 h/day.

2.3. Plasma preparation

Twenty-one female fish (approximately 500 g) were killed by a blow to the head and blood was taken from the caudal artery by means of pre-cooled syringes containing 0.1% heparin (5000 IU/ml, Sigma) and 1% of the protease inhibitor Aprotinin (10 TIU/ml, Sigma, St. Louis, MI, USA) and centrifuged at 2000 × *g* (10 min at 4 °C). The supernatant was carefully decanted, the samples pooled and immediately frozen in aliquots at −80 °C for subsequent binding assays.

2.4. Competitive binding assay

The binding of test compounds to the rtSBP was assessed under competitive conditions (competitive binding studies) essentially as described by Tollefsen (2002). In summary, plasma samples from female rainbow trout were thawed and diluted (0.2 mg protein/ml) in 50 mM phosphate buffer, pH 7.4, containing 0.1% gelatin (PG-buffer). Aliquots (200 μl) were incubated with 100 μl of 2.5 nM [^{3}H]-E2 (diluted in PG-buffer) in combination with increasing concentrations of different unlabeled test chemicals (25 nM–250 μM) for 16 h (4 °C). Incubation was terminated by adding 200 μl ice-cold DCC-solution (2.5% charcoal and 0.25% dextran in PG-buffer) and incubated for 5 min. The DCC-bound steroids were removed by centrifugation at 2000 × *g* (5 min at 4 °C). Each sample was assayed in triplicate and kept on ice during all of the above steps. Aliquots of supernatant (300 μl) were added to 3 ml Ultima Gold liquid scintillation cocktail (Packard, Groningen, The Netherlands) and counted under standard tritium conditions. The difference between total (2.5% vehicle methanol alone) and non-specific binding (1000-fold excess of unlabeled steroid) represents the specific plasma steroid binding. IC_{50} values of the test compounds were calculated as the concentration that caused 50% inhibition of [^{3}H]-E2 binding and determined by non-linear regression using GraphPad Prism 4.0 software (GraphPad software Inc. San Diego, California, USA). Relative binding affinities (RBA) were determined by comparing the obtained IC_{50} values for the test compound relative to that of E2. Plasma protein content was determined by the method of Bradford (1976) after modifying for micro-plates as described by Tollefsen (2002).

3. Results

A range of alkylphenols, alkylated non-phenolics and the natural estrogen E2 were found to successfully bind to the high-affinity estradiol-binding sites in rainbow trout plasma. The use of the vehicle methanol (<1%) did not alter the binding characteristics of the assumed rtSBP (results not shown).

3.1. Size of the alkyl group

The binding affinities of phenol, methylphenol, ethylphenol, an isomeric mixture of 4-dodecylphenol and alkylphenols with normal (*n*) alkyl chains ranging from propyl- to nonylphenol to the rtSBP are shown in Fig. 1. The results from these studies show that all alkylphenols, with the exception of phenol and 4-*n*-nonylphenol, were able to bind successfully to the rtSBP in a dose-dependent manner. The binding affinities for the alkylphenols were found to be fairly similar for most chemicals, although 4-*n*-nonylphenol displayed about 1000 times less binding affinity for the rtSBP than the most potent AP, 4-*n*-butylphenol. The rank order of affinity was 4-*n*-butylphenol > 4-*n*-octylphenol > 4-dodecylphenol > 4-*n*-hexylphenol > 4-*n*-pentylphenol ≈ 4-*n*-heptylphenol > 4-*n*-propylphenol > 4-ethylphenol ⩾ 4-methylphenol ⩾ 4-*n*-nonylphenol. The affinities of alkylphenols for the rtSBP did not follow a clear relationship with the size of the substituted group. The binding affinities of the tested