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Estrogenicity of alkylphenols and alkylated non-phenolics in a rainbow trout (*Oncorhynchus mykiss*) primary hepatocyte culture

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Abstract

Alkylphenols act as estrogen mimics by binding to and transactivating estrogen receptors (ERs) in fish. In the present study, activation of ER-mediated production of the estrogenic biomarker vitellogenin (vtg) in a primary culture of rainbow trout (*Oncorhynchus mykiss*) hepatocytes was used to construct a structure–activity relationship for this ubiquitous group of aquatic pollutants. The role of alkyl chain length and branching, substituent position, number of alkylated groups, and the requirement of a phenolic ring structure was assessed. The results showed that most alkylphenols were estrogenic, although with 3–300 thousand times lower affinity than the endogenous estrogen 17β-estradiol. Mono-substituted tertiary alkylphenols with moderate (C4–C5) and long alkyl chain length (C8–C9) in the para position exhibited the highest estrogenic potency. Substitution with multiple alkyl groups, presence of substituents in the ortho- and meta-position and lack of a hydroxyl group on the benzene ring reduced the estrogenic activity, although several estrogenic alkylated non-phenolics were identified. Co-exposures with the natural estrogen 17β-estradiol led to identification of additional estrogenic compounds as well as some anti-estrogens. A combination of low affinity for the ER and cytotoxicity was identified as factors rendering some of the alkylphenols non-estrogenic in the bioassay when tested alone.

Keyword: Endocrine disrupters; Estrogen mimics; Anti-estrogen; Xenestrogen; Estrogen receptor; Vitellogenin; Cytotoxicity; Structure–activity relationship; Fish

1. Introduction

Alkylphenols have been found as ubiquitous pollutants in waters worldwide (Blackburn and Waldock, 1995; Petrovic et al., 2002) and are proposed to contribute significantly to the endocrine disrupting potential in natural water (Cespedes et al., 2005; Fenet et al., 2003). Potential adverse impact on these and other groups of environmental estrogens (xenoestrogens) has prompted the development of several in vitro bioassays capable of assessing the endocrine disrupting activity, either as nuclear receptor agonists or antagonists. To date, alkylphenols have primarily been shown to be estrogenic in various in vitro bioassays such as estrogen receptor (ER) binding assays (Knudsen and Pottinger, 1999; Olsen et al., 2005; Tollefsen and Nilsen, 2007), recombinant yeast-reporter systems (Routledge and Sumpter, 1997), estrogen-dependent breast cancer (MCF_7) cells (Olsen et al., 2005; Soto et al., 1995) and in primary cell cultures (Jobling and Sumpter, 1993; Tollefsen et al., 2003). The alkylphenols have also been reported to interfere with thyroid hormone receptor activity (Kudo and Yamauchi, 2005), steroid metabolism (Arukwe et al., 1997), and plasma binding of endogenous steroids in aquatic animals (Tollefsen, 2007), thus indicating that this group of pollutants act through multiple mechanisms to potentially cause endocrine disruption.

The estrogenic activity of alkylphenols has been related to their ability to mimic the natural ligand 17β-estradiol in binding to and activating the ER. Several structure–activity relationship (SAR) and quantitative structure–activity

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relationship (QSAR) models utilizing in vitro tests systems based on the human ER (hER) and fish ERs have identified structural features important for the (anti)estrogenicity of alkylphenols (Hu and Aizawa, 2003; Routledge and Sumpter, 1997; Schmieder et al., 2000b; Tabira et al., 1999; Tollefsen and Nilsen, 2007). Recent studies have also identified that several alkylated non-phenolics resembling the structure of alkylphenols have the potential to act estrogenic in fish (Olsen et al., 2005; Tollefsen and Nilsen, 2007). Interestingly, moderately sized alkylphenols such as butylphenol was equipotent with larger sized alkylphenols such as octylphenol and nonylphenol (Jobling and Sumpter, 1993; Olsen et al., 2005; Tollefsen and Nilsen, 2007), a finding which contrast the estrogenic potency determined by in vitro bioassays based on the hER (Hu and Aizawa, 2003; Routledge and Sumpter, 1997; Tabira et al., 1999). Although minor interspecies differences in the binding activity of a variety of estrogen mimics to the fish ERs have been reported (Denny et al., 2005; Tollefsen et al., 2002; Tollefsen and Nilsen, 2007), larger differences may exist between more evolutionary distant species (Matthews et al., 2000). It is, however, acknowledged that estrogenicity is dependent on ligand-binding affinity, transcriptional and post-transcriptional regulation of ER-dependent genes, and the toxicokinetics of the compounds in the test system (Beresford et al., 2000; Flouriot et al., 1996; Katzenellenbogen et al., 2000), thus emphasizing the importance of determine both ER binding activity, cellular estrogenic responses and reproductive effects in vivo to fully characterize the estrogenic potential of xenoestrogens.

Bioassays based on recombinant cells stably transfected with receptor-reporter gene systems which exhibit high sensitivity, reproducibility and ability to screen large number of samples are routinely in use to determine the endocrine disrupting activity of chemicals. Although these systems exploit the simplicity and robustness of high throughput in vitro bioassays, they normally lack the complexity of intact native cells (Beresford et al., 2000; Olsen et al., 2005; Welshons et al., 2003). Primary cell cultures and tissue preparations from fish, which express the natural populations of ER isoforms and the inherent metabolic machinery of native cells (Flouriot et al., 1995; Pesonen and Andersson, 1991; Schmieder et al., 2000a), have been utilized with success to assess multi-endpoint toxicity of single chemicals, complex mixtures and environmental samples (Finne et al., 2007; Schmieder et al., 2004; Smeets et al., 1999a; Tollefsen et al., 2006) and represent a versatile alternative to the use of recombinants bioassays.

In the present work, a SAR approach has been adopted to assess the estrogenic activity of alkylphenols and determine which structural requirements that are associated with estrogenicity in native fish cells. In this approach, ER-mediated induction of vitellogenin (vtg) production was used as a measure of estrogenicity in primary hepatocytes from rainbow trout (*Oncorhynchus mykiss*). The hepatic induction of vtg production in male and juvenile oviparous fish, which normally only occur in maturing females under stimulation of 17β-estradiol, has been proposed as a sensitive biomarker for the exposure to estrogenic chemicals of endogenous and exogenous origin (Sumpter and Jobling, 1995). The induction of vtg production has been utilized for studying the estrogenic action of pure chemicals and a suitable biomarker for the exposure of environmental estrogens in vivo and in vitro (Andersen et al., 1999; Knudsen et al., 1997; Tollefsen et al., 2003), and is proposed as a core endpoint in OECD testing for endocrine disruption in fish (OECD, 2004). The cytotoxicity of the alkylphenols was measured simultaneously since it may affect the interpretation of the in vitro estrogenicity caused by xenoestrogens (Smeets et al., 1999b; Welshons et al., 2003).

2. Materials and methods

2.1. Chemicals

The test chemicals copper sulfate ($CuSO_4 \times 5H_2O$), 17β-estradiol, 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-*i*-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*-butylbenzoic acid, 4-*t*-butylbenzaldehyde, 4-*n*-butylchlorobenzene, 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). All chemicals (purity $\geqslant$96%) were diluted in anhydrous ultra pure DMSO (99.9%, Sigma-Aldrich) or directly in growth media ($CuSO_4$) prior to use. Chemicals in DMSO were stored in the dark at $-20\,^{\circ}C$ until use.

2.1.1. Fish

Immature male rainbow trout (200–500 g), obtained from Killi Oppdrettsanlegg (Dombås, Norway), were kept in tanks at the Institute of Biology, University of Oslo (Norway), at a water temperature of 8–10 °C, oxygen saturation of approximately 100% and pH 6.6. The fish were fed daily with commercial fish pellets (EWOS, Bergen, Norway) in amounts corresponding to 0.5% of total body mass until isolation of the liver cells. The tanks received artificial illumination (100 lux) for 12 h/day.

2.2. Hepatocyte isolation, culturing, and exposure

Hepatocytes were isolated, cultured and exposed essentially as described by Tollefsen et al. (2003). At sampling, fish was killed by a blow to the head and the liver was perfused *in situ* (10 ml/min, 10 min) with a calcium-free solution containing: NaCl (122 mM); KCl (4.8 mM); $MgSO_4$ (1.2 mM); Na_2HPO_4 (11 mM); NaH_2PO_4 (3.3 mM); $NaHCO_3$ (3.7 mM) and EGTA (26 μM) at 2 °C, until all blood had been washed out. The liver was then perfused for 10–15 min with the same buffer, now containing $CaCl_2$ (1.5 mM) instead of EGTA, and 0.3 mg/ml collagenase (Type IV, Sigma-Aldrich) at 37 °C to maximize the enzymatic tissue digestion. The liver was then removed and the cells dispersed in ice-cold calcium free buffer (EGTA-buffer) containing 0.1% (w/v) BSA (Sigma-Aldrich). The cell suspension was filtered through 250 μm and then a 100 μm nylon mesh and centrifuged at 50g for 3 min at 4 °C. The cells were washed with ice cold EGTA-buffer three times before finally to be resuspended in serum-free L-15 medium containing L-glutamine (0.29 mg/mL), $NaHCO_3$ (4.5 mM), penicillin (100 Units/mL), streptomycin (100 μg/L) and amphotericin (0.25 μg/mL), all supplied by Cambrex