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Estrogen-related receptor γ disruption of source water and drinking water treatment processes extracts

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Abstract

Environmental chemicals in drinking water can impact human health through nuclear receptors. Additionally, estrogen-related receptors (ERRs) are vulnerable to endocrine-disrupting effects. To date, however, ERR disruption of drinking water potency has not been reported. We used ERRγ two-hybrid yeast assay to screen ERRγ disrupting activities in a drinking water treatment plant (DWTP) located in north China and in source water from a reservoir, focusing on agonistic, antagonistic, and inverse agonistic activity to 4-hydroxytamoxifen (4-OHT). Water treatment processes in the DWTP consisted of pre-chlorination, coagulation, coal and sand filtration, activated carbon filtration, and secondary chlorination processes. Samples were extracted by solid phase extraction. Results showed that ERRγ antagonistic activities were found in all sample extracts, but agonistic and inverse agonistic activity to 4-OHT was not found. When calibrated with the toxic equivalent of 4-OHT, antagonistic effluent effects ranged from 3.4 to 33.1 μg/L. In the treatment processes, secondary chlorination was effective in removing ERRγ antagonists, but the coagulation process led to significantly increased ERRγ antagonistic activity. The drinking water treatment processes removed 73.5% of ERRγ antagonists. To our knowledge, the occurrence of ERRγ disruption activities on source and drinking water *in vitro* had not been reported previously. It is vital, therefore, to increase our understanding of ERRγ disrupting activities in drinking water.

Key words: drinking water; estrogen receptor; estrogen-related receptor; two-hybrid yeast; solid phase extraction

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Introduction

Over the last several decades, an increasing number of environmental contaminants have been found to disrupt endocrine systems in wildlife and humans (Sonnenschein and Soto, 1998). These endocrine disrupting chemicals (EDCs) can interact with human nuclear receptors (NRs), interfering with the endocrine system, causing developmental degeneration, reducing fecundity, and leading to an increase in human breast cancer (Colborn et al., 1993; Crews et al., 2000). Recently, EDCs have emerged as a major water quality concern as they can interfere with endocrine systems when found at certain concentrations in drinking water (Scruggs et al., 2005). Many EDCs are biologically active at very low concentrations and have been detected in surface water (Heberer et al., 2002) and drinking water (Stackelberg et al., 2004). This is of concern as many conventional treatment processes are ineffective in completely removing EDCs from water (Johnson et al., 2007).

While research has focused on the disruption activity of estrogen receptors (ERs) in water in recent years, far less attention has been paid to identifying compounds with estrogen-related receptors (ERRs) disrupting activity. Increasing evidence from *in vivo* and *in vitro* studies demonstrates, however, that ERRs are vulnerable to endocrine-disrupting effects (Horard and Vanacker, 2003) and are possibly disrupted by environmental chemicals (Takayanagi et al., 2006). Recent research has also documented a correlation between ERs and ERRs in breast cancer patients (Ariazi et al., 2002), indicating that ERR disrupting chemicals may play an important role in breast tumors. Therefore, the rate of elimination of ERRs disrupting substances during the treatment process of drinking water and assessing the disrupting potency of surface water resources are of considerable environmental importance.

Since their identification in the 1990s, ERRs have been classified as an orphan nuclear receptor subfamily as no endogenous ligands have been identified. Both ERRs and ERs have a high degree of amino acid sequence similarity and identity in their DNA-binding (DBD) and

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ligand-binding (LBD) domains (Horard and Vanacker, 2003). ERRs can bind to functional estrogen response elements (EREs) in ERs target genes, suggesting possible similarities between ERRs and ERs action (Takayanagi et al., 2006). ERRγ and ERs functional crosstalk systems might explain low-dose effects of environmental estrogen bisphenol A (Takayanagi et al., 2006). Of the three ERR types, ERRα, ERRß and ERRγ (Giguere et al., 1988; Hong et al., 1999), ERRγ is essential for the development of the hypothalamic-hypophyseal-adrenocortical axis (Wilson et al., 1993; Luo et al., 1994), and is expressed in a number of human adult and fetal tissues including the brain, skeletal muscle, heart, kidney, and retina. Some synthetic chemicals including bisphenol A, diethylstilbestrol, 4-nonylphenol and some phytoestrogens can bind to human ERRγ (Tremblay et al., 2001; Coward et al., 2001; Greschik et al., 2004; Takayanagi et al., 2006), which have been detected in many environment samples and drinking water. Assessing the environmental pollutants interfering with ERRγ is, therefore, of great importance.

We previously developed novel screening methods for chemicals with ERRγ disrupting properties using a yeast two-hybrid system, and found that some pesticides had ERRγ disrupting activity (Li et al., 2008a). Although many endocrine disrupting chemicals can survive drinking water treatment (Westerhoff et al., 2005), little is known about the fate of ERRγ disrupting chemicals in drinking water and their possible effects on human health. In the present study we assessed, therefore, the ERRγ mediated effects in source and drinking waters using the yeast two-hybrid system. As ERRγ shows very high constitutive activity without ligand addition, the disrupting activities of drinking water extracts were tested with and without the standard antagonist4-hydroxytamoxifen (4-OHT) (Takayanagi et al., 2006) using ERRγ-GRIP1 yeast by measuring the change of β-galactosidase activity.

1 Materials and methods

1.1 Chemicals

High performance liquid chromatography (HPLC) grade dichloromethane, hexane, methanol, and tert-butyl methyl ether were obtained from Fisher Scientific (Fair Lawn, USA). The 4-hydroxytamoxifen (4-OHT, 98%) and dimethyl sulfoxide (DMSO, 99.5%) were obtained from Sigma Chemical (USA). For all chemicals, stock solutions were prepared in DMSO.

1.2 Sample collection and processing

Sampling was conducted in May 2007 at a drinking water treatment plant (DWTP) in north China. The DWTP had four treatment lines with a total capacity of 1,500,000 m^3 per day. Samples were collected from line two, which processed the source water from a reservoir. The DWTP consisted of pre-chlorination, coagulation, coal and sand filtration, activated carbon filtration, and secondary chlorination (Fig. 1).

Each water sample (20 L) was collected in a pre-cleaned amber glass bottle. The bottle was washed three times with deionized water before sample collection. Approximately 2 mL/L (V/V) of methanol was added to each sample immediately after sampling to suppress possible biotic activity. All samples were stored at 4°C and treated within 8 hr after sampling.

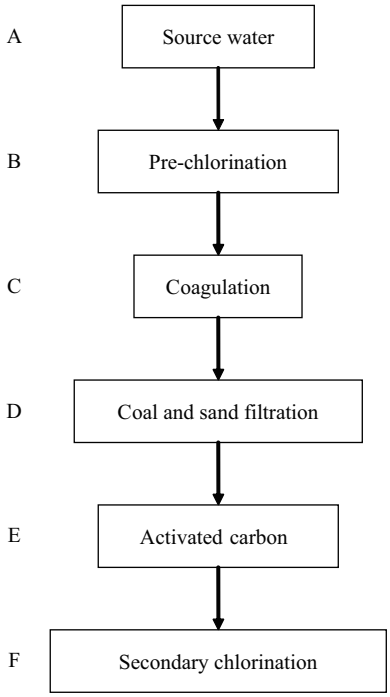

Fig. 1 Flow scheme of the treatment processes and sampling locations (A–F).

Water samples and procedural blank (Mini-Q water, 18.2 Ω) were filtered with glass fiber filters (0.45 μm, Whatman, England) to remove insoluble materials. Solid phase extraction (SPE) was then performed using 500 mg Oasis HLB cartridges (Waters, USA) conditioned according to the manufacturer's directions. The cartridges were forced under vacuum at a flow rate of approximately 6 mL/min. The cartridges were kept under vacuum aspiration for 5 min to dry off any residual water, and then washed twice with 5 mL of hexane/dichloromethane (7/3, V/V), twice with 5 mL of tert-butyl methyl ether, twice with 5 mL of dichloromethane/methanol (9/1, V/V), and once with 5 mL of methanol at a flow rate of 1 mL/min. The extracts were then combined and filtered by anhydrous sodium sulphate to remove water and evaporated to dryness in a rotary evaporator (R-200, Buchi, France) at 40°C to 2 mL. The dehydrated extract was then dried under gentle nitrogen flow and reconstituted in 0.1 mL of DMSO and used for bioassay immediately.

1.3 Bioassay

The bioassay was conducted using yeast strain hERRγ-GRIP1 (Li et al., 2008a). All assays were conducted in at least triplicates. Each assay group included the sample, the positive control (4-OHT for ERR antagonistic activity), the negative control (DMSO), and the procedural blank. Test samples (5 μL) of serial dilutions were combined with 995 μL of medium containing 5×10^3 yeast cells/mL, resulting in a test culture in which the volume of DMSO did not

exceed 1.0% of the total volume. Control assays were performed for all field blank and laboratory blank samples and found to be lower than the detection limit.

Test cultures (200 μL) were transferred into each well of a 96-well plate and incubated at 30°C with vigorous orbital shaking (800 r/min) on a titer plate shaker (Heidolph TITRAMAX 1000, Hamburg, Germany) for 2 hr. The cell density of the culture was then measured at 600-nm wavelength (TECAN GENios A-5002, Salzburg, Austria). After that, 50 μL of test culture was transferred to a new 96-well plate and after addition of 120 μL of Z-buffer ((in g/L) $Na_2HPO_4{\cdot}7H_2O$ 16.1; $NaH_2PO_4{\cdot}H_2O$ 5.5; KCl 0.75; $MgSO_4{\cdot}7H_2O$ 0.246) and 20 μL of chloroform, the assays were carefully mixed (vortex 25 sec) and preincubated for 5 min at 30°C. The enzyme reaction was started by adding 40 μL of o-nitrophenyl-β-D-galactopyranoside (13.3 mmol/L, dissolved in Z-buffer), then incubated at 30°C on a titer plate shaker for 60 min. The reactions were terminated by the addition of 100 μL of Na_2CO_3 (1 mol/L). After centrifugation at 12,000 ×g for 15 min (Sigma Laborzentrifugen 2K15, Osterode, Germany), 200 μL of the supernatant was transferred into a new 96-well plate and the OD_{420} nm was determined.

The β-galactosidase activity (u) was calculated according to the following Eqs. (1) and (2):

$$u = C_S/t \times V \times D \times \mathrm{OD_S} \quad (1)$$

$$C_S = 10^{-6}(A_S - A_B) / \varepsilon \times d \quad (2)$$

where, C_S: the concentration of o-nitrophenol in the enzyme assay reaction mix, t: incubation duration of the enzyme reaction, V: volume of the test culture, D: dilution factor, OD_S: OD_{600} of test culture, A_S: OD_{420} of the enzyme supernatant of the sample, A_B: OD_{420} of the enzyme reaction supernatant of the blank, ε: ε for o-nitrophenol in the enzyme assay reaction mix, and d: diameter of the cuvette (Routledge and Sumpter, 1996; Gaido et al., 1997; Li et al., 2008b).

To exclude false results caused by cytotoxicity, cell viability was determined spectrophotometrically as a change in cell density (OD_{600}) in the assay medium. The procedural blank was tested at the same concentration to monitor for a false-positive result. Detailed steps are described elsewhere (Li et al., 2008c).

1.4 Data analysis

Results were performed on the toxic equivalent (TEQ) approach (Qiao et al., 2006). The dose-response curve on the inhibition of β-galactosidase expression by 4-OHT is described in our previous work (Li et al., 2008a). The extract responses were calibrated according to the dose-response curve of 4-OHT to obtain the bioassay derived equivalent concentrations (TEQ). If necessary, the extract was diluted to fit the linear part of the dose-response curve for 4-OHT.

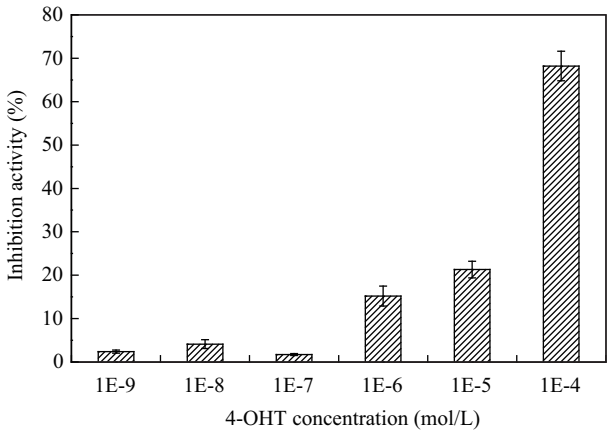

Fig. 2 Inhibition of high constitutive β–galactosidase activity by 4-hydroxytamoxifen (4-OHT) in yeast strain ERRγ-GRIP1. Chemical antagonist activity is represented as inhibition activity percentage relative to high constitutive activity in yeast strain ERRγ-GRIP1. Values are average ± standard error (n = 3).

2 Results and discussion

2.1 Response to known ERRγ antagonist 4-OHT

The antagonistic activity of varying 4-OHT concentrations was measured (Fig. 2). The 4-OHT antagonistic activity in a concentration-dependent manner was similar to that previously reported (Li et al., 2008a). The half maximal effective concentration (EC50) value of 4-OHT was 8.0×10^{-6} mol/L.

2.2 Response to drinking water extracts

Drinking water extracts did not increase β-galactosidase activity in the yeast assay compared with the negative control. However, all extracts had ERRγ antagonistic activities that inhibited β-galactosidase expression. Removal rates of ERRγ antagonistic activities of B, C, D, E, and F treatment processes were –28.9%, –155.0%, –60.3%, –44.7% and 73.5% at fifty-fold raw water concentration compared with the reservoir water (step A) (Fig. 3). When calibrated regarding the toxic equivalent (TEQ) of 4-OHT (Fig. 2), values ranged from 3.4 to 33.1 μg/L 4-OHT (Fig. 4).

After tested samples were incubated with 4-OHT (1.0 ×

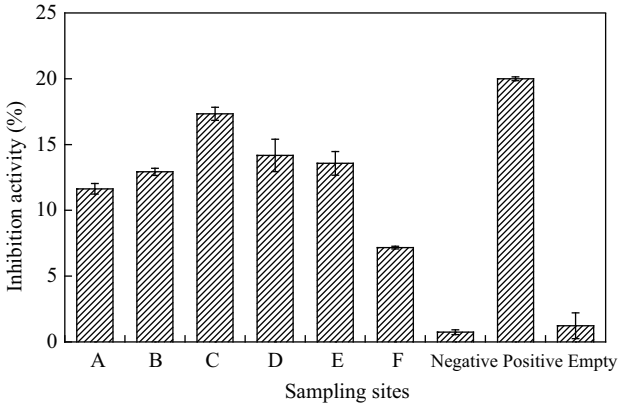

Fig. 3 ERRγ antagonistic activity of the sample in yeast strain ERRγ-GRIP1 (fifty times concentrated of raw water). Values are average ± standard error (n = 3). Negative: dimethyl sulfoxide (DMSO); Positive: 4-OHT; Empty: procedural blank.

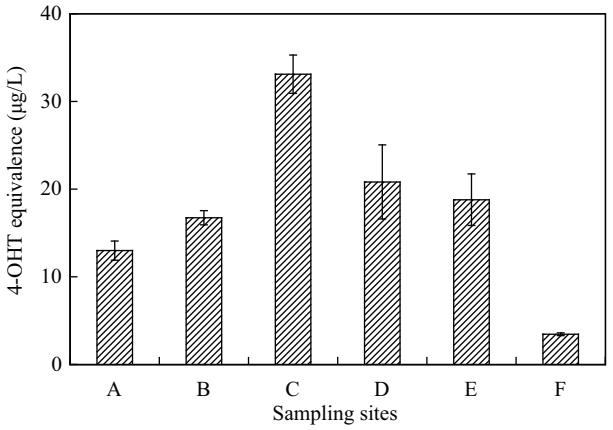

Fig. 4 Bioassay-derived 4-OHT equivalence in yeast strain ERRγ-GRIP1 for sample ERRγ antagonistic activity. Values are average ± standard error (n = 3).

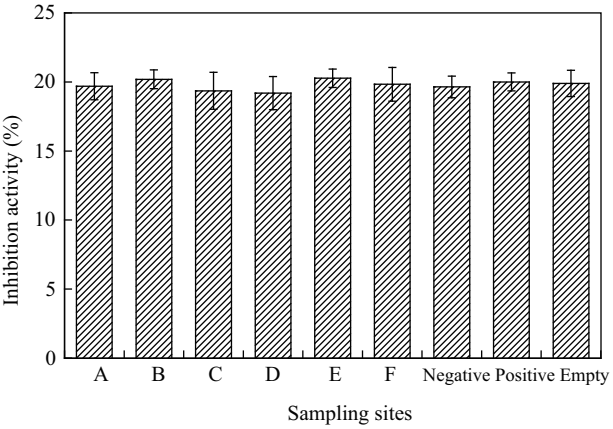

Fig. 5 Effect of test samples on inverse agonist activity of 4-OHT in yeast strain ERRγ-GRIP1 for ERRγ antagonistic activity at fifty-fold concentration. Sample inverse agonist activity is represented as inhibition activity percentage relative to the high constitutive activity in yeast strain ERRγ-GRIP1. Values are average ± standard error (n = 3). Negative: dimethyl sulfoxide (DMSO), Positive: 4-OHT.

10^{-5} mol/L), which inhibited about 20% ERRγ constitutive activity in the recombined yeast, 4-OHT's inverse activity was not suppressed (Fig. 5).

2.3 Discussion

Multiple contaminants such as pharmaceuticals, steroid hormones, unregulated pesticides, flame retardants, rocket fuel chemicals, plasticizers, detergents, and stain repellants have been found in source and drinking waters (Kolpin et al., 2002; Barnes et al., 2008; Focazio et al., 2008). Although they are at low levels in water, considering only trace amounts of natural hormones can affect the body, there is growing interest in understanding the fate of EDCs during drinking water treatment (Benotti et al., 2009). As contaminant removal by applied water treatment is often incomplete, natural waters contain many dissolved chemicals that affect ecosystems and impact drinking water supplies (Boyd et al., 2003).

In the present work, ERRγ antagonistic activities were found in the reservoir and DWTP. When calibrated to the TEQ of 4-OHT, values ranged from 3.4 to 33.1 μg/L. The ERRγ antagonistic activities found in reservoir source water with 4-OHT equivalent was 13.0 μg/L, suggesting that source water contained many compounds that bind to ERRγ. Among the processes, secondary chlorination was effective in removing ERRγ disrupting substances, but coagulation led to a significant increase in ERRγ disrupting activity. Many previous studies have found that coagulation, flocculation, and precipitation were ineffective at removing dissolved organic contaminants, especially for low molecular weight compounds (Ternes et al., 2002). Additionally, pre-chlorination was responsible for higher concentrations of disinfection byproducts including many organic chemicals (Simpson and Hayes, 1998), and coagulation caused small organic molecules to increase (Luo et al., 1998). In the present study, activated carbon showed no obvious removal effect. Although activated carbon has been effective at removing organic contaminants, removal capacity was limited by contact time, competition from natural organic matter, contaminant solubility, and carbon type (Kolpin et al., 2002; Boyd et al., 2003). Other studies have also stated that several compounds were detectable in carbon effluent, and removal efficiency of activated carbon was largely dependent on water quality (Snyder et al., 2007). Compared with source water, DWTP can remove 73.4% of ERRγ disrupting substances, indicating that ERRγ antagonist elimination in the DWTP was incomplete. Considering some ERRγ disrupting substances remained in the final effluent, it is possible that the outlet might be harmful to human health.

An increasing number of organic compounds have been detected in surface waters, raising concerns about the contamination of water resources as it is sometimes necessary to produce drinking water from polluted surface waters (Heberer et al., 2002). However, complete removal of EDCs is not possible through conventional wastewater treatment and a significant fraction of EDCs may be released into the aquatic environment (Halling-Sorensen, 1998; Daughton and Ternes, 1999; Joss et al., 2004). The discharge of ERRγ disrupting chemicals in water must, therefore, be understood.

Considering the important role ERRs play in human health (Ariazi et al., 2002), assessing the rate of elimination of ERR disrupting substances during DWTPs is of considerable importance. To date, however, no report on ERR disrupting activity in DWTPs has been presented. Results from this study suggested that ERRγ antagonistic activity in drinking water may biologically impact humans, and ERRγ antagonists in drinking water acting via more than one mechanism might contribute to biological effects on organisms and contribute to a wide range of hormonal and/or anti-hormonal effects *in vivo* through different pathways (Molina-Molina et al., 2006). Although ERRs play an important role in breast cancer (Ariazi et al., 2002), for example, current information is insufficient for determining whether ERRγ antagonistic activity levels in drinking water can affect the human endocrine system. Further research is needed, especially *in vivo* studies, to assess the risk of drinking water on endocrine disruption in humans. Based on current knowledge, it is clear that ensuring a safe and sustainable water supply will be an increasingly great challenge. In addition, drinking water

companies must effectively reduce the concentration of all contaminants to guarantee clean drinking water for consumers (Knepper et al., 1999).

3 Conclusions

This study described ERRγ disrupting activities in reservoir and drinking water treatment processes. Secondary chlorination was effective in removing ERRγ antagonists, while coagulation led to a significant increase in ERRγ antagonistic activity. The waterworks processes were able to remove 73.5% of ERRγ antagonists compared to source water, indicating that there are concerns about ERRγ disrupting contaminants in drinking and source waters. The ERRγ two-hybrid yeast assay can be employed as an important and useful method for drinking water safety evaluation, allowing ERRγ disrupting pollutants to be detected efficiently and accurately.

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