Causes of endocrine disrupting potencies in surface water in East China

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HIGHLIGHTS

- TR and AR antagonist equivalents were detected in grade II surface water for the first time.
- The greatest TR and AR antagonistic potency was detected in the Yangtze River being greater than LOEC.
- Diisobutyl phthalate and dibutyl phthalate accounted for most effects to TR antagonistic potency.
- PAEs explained little of AR antagonistic equivalents, being different from previous studies.
- Octylphenol, nonylphenol and benzo[a]pyrene might be responsible for the AR antagonistic potency.

ARTICLE INFO

ABSTRACT

Surface water is essential for human health and ecological diversity, but some endocrine disrupting chemicals are detectable. Both thyroid receptor (TR) and androgen receptor (AR) agonistic/antagonistic potencies in grade II surface water in East China were investigated using reporter gene assays. While none of the water exhibited agonistic potency, significant AR and TR antagonistic potencies were detectable. TR antagonistic equivalents (TR-AntEQ) and AR antagonistic equivalents (AR-AntEQ) ranged from 3.6 to 76.1 μg dibutyl phthalate/L and from 2.3 to 242.6 μg flutamide/L, respectively. The TR and AR antagonistic potencies in the Yangtze River watershed were highlighted, with equivalents greater than the lowest observable effect concentration (LOEC) of dibutyl phthalate and flutamide, respectively. Phthalate esters (PAEs) being the most abundant explained most of the TR antagonistic potency, contributing more than 65% of the TR-AntEQ and diisobutyl phthalate (DiBP) was the major contributor. In most surface waters studied, PAEs contributed little of the AR-AntEQ, but the frequently detected octylphenol, nonylphenol and benzo[a]pyrene might be responsible.

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1. Introduction

Over the past 20 years, a variety of synthetic chemicals which are able to interact with different nuclear receptors, mimic or counteract natural hormones to produce pharmacological effects and further interfere with the endocrine systems have been receiving increasing attention (Benotti et al., 2008; Li et al., 2012). These endocrine disrupting compounds (EDCs) might trigger alterations at extremely small concentrations and increase their potential risks to humans and wildlife (Bellingham et al., 2009). Most previous studies have especially focused on potencies of mixtures and individual chemicals contributing to estrogenic hormone

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potencies (Martínez-Gómez et al., 2013), while limited information is available for disruptions of thyroid hormone (TH) and androgenic hormone (AH) potencies (Gong et al., 2014). Androgens are essential for sexual differentiation and maintenance of the reproductive tract of males (Juntti et al., 2010). Thyroid hormone is responsible for normal development of the brain in higher vertebrates and postembryonic development of lower vertebrates (Howdeshell, 2002). Disruption of TH or AH might cause irreversible effects, such as mental retardation, neurological deficits and testicular cancers (Moriyama et al., 2002). Some environmental pollutants have been reported to interact with androgenic receptor (AR) or thyroid receptor (TR) by inhibiting binding to its endogenous ligands or by providing additional ligands that can bind to the receptor (Lee et al., 2003; Shen et al., 2009) and might pose a risk to human health.

The Huai River (Ch: *Huaihe*), Tai Lake (Ch: *Taihu*) and lower reaches of the Yangtze River (Ch: *Chang Jiang*) are among the most important waters in East China and are characterized by heavy industry and chemical industrial parks. In recent years, due to rapid development of petroleum and petrochemical industries, a variety of organic contaminants, which might disrupt endocrine function, have been discharged into these three waters (Shen et al., 2006). Previous studies have also reported the agonistic/antagonistic potencies of mixtures for AR or TR detected in drinking water sources in East China (Hu et al., 2013; Li et al., 2010), although the responsible chemicals are still unknown. The results indicated that AH and TH disrupting chemicals exist in water sources, although they are always extremely protected. Concerns about these contaminants warrant comprehensive monitoring of TH and AH disrupting potencies in other less protected aquatic environments and identification of individual chemicals contributing to these potencies of mixtures.

Surface waters in China are classified into five grades according to their designated uses. Grade I surface waters, of which there are few in East China, are protected most and are normally headwaters, sources of drinking water and national protected zones. Grade II surface waters supply water sources and special areas to protect rare and endangered species (Shi et al., 2013a). Grade II surface waters are more common and this classification is considered to be the cleanest aquatic environments in East China. Grade I or grade II surface waters can serve as source water. According to the national standards, the number of conventional indicators of the grade I or grade II surface waters include chemical oxygen demand (COD), biochemical oxygen demand (BOD), and dissolved oxygen (DO), is much lesser than the indicators of the water sources which include concentrations of additional chemicals. Detectable concentrations of polycyclic aromatic hydrocarbons (PAHs), pesticides and alkylphenol have also been found in some grade II surface waters (Shi et al., 2013a). These chemicals have been reported to induce both anti-AR and anti-TR activities, and may pose risks to the safety of surface water (Floehr et al., 2013). Therefore, it was deemed that more research was needed to assess concentrations of AH and TH disrupting potential in grade II surface waters of east China.

Phthalate esters (PAEs), because of the greater concentrations compared with other contaminants, have been demonstrated previously to be major contributors to observed AR and TR (ant)agonist potencies in water sources and tap water (Hu et al., 2013; Shi et al., 2012). However, it is still unknown whether or not PAEs were primary contributors to endocrine disrupting potencies in grade II surface waters. There are several PAEs: di-2-ethylhexyl phthalate (DEHP), dibutyl phthalate (DnBP), dimethyl phthalate (DMP), diethyl phthalate (DEP), benzyl butyl phthalate (BBP) and di-n-octyl phthalate (DNOP) being the most commonly produced (Dargnat et al., 2009). PAEs are used as plasticizers in a variety of materials, including food packaging, plastic toys, medical devices, bags, clothing, insect repellent, vinyl flooring and polyvinyl chloride (PVC) tubing (Bornehag et al., 2004; Janjua et al., 2007).

In the present study, *in vitro*, reporter gene, transactivation assays and instrumental analysis were employed to assess the TR and AR (ant)agonist potencies in grade II surface water as well as concentrations of specific chemicals that could contribute to the observed potencies. These locations were suspected to have been affected by human activities, including industrial production. Objectives of this study were to: 1) Detect TR and AR agonist and antagonist potencies in water; 2) Identify the potential responsible compounds for TR and AR (ant)agonist potencies of extracts by use of a combination of instrumental analysis and bioassays; 3) Examine contributions of the pollutants and potential adverse effects in grade II surface waters.

2. Material and methods

2.1. Chemicals

Phthalate esters including di-2-ethylhexyl phthalate (DEHP), dibutyl phthalate (DnBP), dimethyl phthalate (DMP), diethyl phthalate (DEP), benzyl butyl phthalate (BBP) and Diisobutyl phthalate (DiBP) of purity >99.5% were purchased from Labor Dr. Ehrenstorfer-Schäfers (Augsburg, Germany). Chemicals including 3-(4,5-dimethylthiazol-2-ol)-2,5-diphenyltetrasodium bromide tetrazolium (MTT) and $_L$-3,5,3′-triiodothyronine (T_3) with the purity of over 97%, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 5α-dihydrotestosterone (DHT) and flutamide with the purity of over 97% was purchased from Labor Dr. Ehrenstorfer-Schäfers.

2.2. Selection of locations and collection of samples

Grade II surface water was collected from lower reaches of the Yangtze and Huai Rivers and Tai Lake in East China. These sources provide drinking water to 80 million residents. The areas selected were known or suspected to be contaminated with wastewater from industries or municipal wastewater treatment plants (WWTP). Samples of water were collected from twelve grade II surface water zones (Fig. 1), during August 2011. To exclude contamination of samples by endocrine disrupting chemicals during the sampling procedure, blanks were prepared with boiled Milli-Q water, which has been proved to contain less concentrations of PAEs than Milli-Q water (Shi et al., 2012).

Untreated water samples from grade II surface water (20 L, half for bioassay and half for chemical analysis) were collected in brown glass bottles that had been precleaned and rinsed with high-purity solvent including hexane (Merck, Darmstadt, Germany), dichloromethane, acetone and methanol (Tedia Co. Ltd, Fairfield, OH, USA), and milli-Q water. Samples of water were stored on ice during transportation, stored at 4 °C in laboratory and extracted within 24 h.

2.3. Sample preparation and instrumental analysis

Samples were extracted by use of a previously reported method with modification (Shi et al., 2013b). Briefly, samples of unfiltered water were extracted by use of tandem C18 cartridges (500 mg/6 mL, glass, Dikma Technology, China) under vacuum at a flow rate of 6–8 mL/min. Cartridges were pre-conditioned with hexane, dichloromethane, acetone, methanol and milli-Q water. For each location, approximately 2 L of water was passed sequentially, through two cartridges in tandem. Then a gentle stream of nitrogen gas was used to reduce the water in cartridges to dryness. Absorbed contaminants were eluted from cartridges sequentially with 10 mL hexane, 10 mL hexane and dichloromethane (4:1, v/v), and then 10 mL dichloromethane and methanol (1:1, v/v). Eluates of
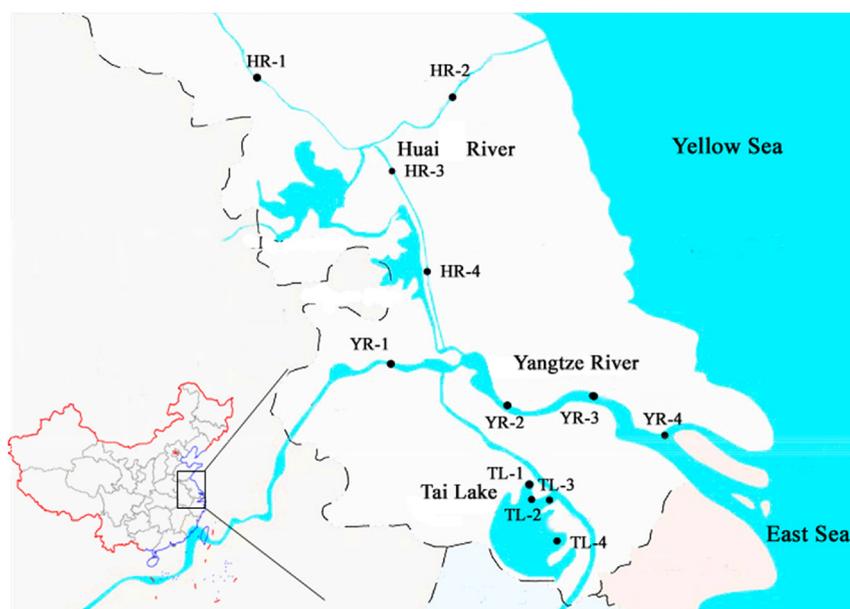
Fig. 1. Map showing locations of sampling locations in East China.

samples from each site were combined into a composite sample and were evaporated by rotary evaporation (type TVE-1000, EYELA, Tokyo, Japan) in a thermostatic bath. Concentrated eluates were separated into two aliquots for use in bioassays and instrumental analysis, respectively. Dehydrated extracts were blown nearly to dryness under a gentle nitrogen flow and reconstituted in 0.2 mL dichloromethane for chemical analysis. Extracts used in bioassays were prepared by reduction nearly to dryness and were reconstituted in 0.2 mL of dimethyl sulfoxide (DMSO), after which they were diluted with appropriate culture medium at 6.25, 12.5, 25, 50, 100 or 200 times the original concentration in raw water.

A Thermo TSQ Quantum Discovery triple-quadrupole mass spectrometer (San Jose, CA, USA), operated in selected-reaction monitoring (SRM) mode, was used for quantitative analysis of PAEs. Helium was used as carrier gas and flow rate was set at 1.0 mL/min. A pulsed splitless injector was used for injecting 1.0 μL. An Rtx-5MS column (30 m × 0.25 mm, film thickness 0.25 μm) was used. Temperature of the inlet was set as 250 °C. Mean recoveries and limits of quantification (LOQ) are given in Table S1. External standards were used to determine recoveries of detected compounds. Furthermore, internal standards including di-n-butyl phthalate-d4, and bis(2-ethylhexyl)phthalate-3,4,5,6-d4 were added to the tested extracts before instrumental analysis for quality control.

Qualitative analyses of target analytes thought to contribute to TR and AR agonistic and antagonistic potencies in samples of water were conducted using GC-MS/MS (Thermo, San Jose, CA, USA) and LC-MS (Thermo, San Jose, CA, USA) in SRM mode and full scan mode.

2.4. Cytotoxicity

Cytotoxicities of organic extracts of water were assessed using the MTT assay according to a previously described protocol (Du et al., 2010). CV-1 cells and MDA-kb2 cells were plated into each well of 48-well or 384-well plates using DMEM with 10% dextran-coated charcoal (DCC) serum at a density of 1 × 10^4 cells/100 μL. After 24 h incubation, MDA-kb2 and CV-1 cells were treated with vehicle solvent or extracts alone or with 5 × 10^{-9} M T$_3$ or 1.0 × 10^{-9} M DHT. After 24 h, MTT (5 mg/mL in PBS, sigma–Aldrich, St. Louis, MO, USA) was added to the plate and contin-

uously incubated at 37 °C for 4 h. Absorbance was measured at 570 nm. The reporter gene assay was conducted only for dilutions that caused no cytotoxicity.

2.5. Reporter gene assay

2.5.1. Reporter gene assay for androgenic receptor

The MDA-kb2 cell line (ATCC CRL-2713), is stably transformed with murine mammalian tumor virus (MMTV)-luciferase was provided by the Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada. Cells were cultured in L-15 medium (Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (FBS, Gibco, Invitrogen Corporation, Carlsbad, CA, USA) at 37 °C in an ambient atmosphere without CO$_2$. Media was supplemented with 10% charcoal-dextran-stripped FBS (CDS-FBS, Biological Industries Ltd. Israel) instead of 10% FBS in the bioassay to minimize background potency. Cells were cultured in assay media for at least 24 h and then plated at 1 × 10^5 cells per mL in 384-well plate (Corning Inc., Corning, NY, USA) with 80 μL of assay media per well. When cells were incubated for 24 h, solvent-control and tested extracts (for AR agonistic potency) or supplemented with 1.0 × 10^{-9} M DHT (for AR antagonistic potency) were added into corresponding wells. The final concentration of DMSO in medium was less than 0.1%. DHT and flutamide were chosen as positive controls for AR agonistic and antagonistic potencies, respectively. Exposure medium was removed after 24 h, and 10 μL of 1 × lysis buffer (Promega, Madison, WI, USA) was added per well. Luciferase reagent (25 μL) was added per well after 10 min for cell lysis, and luminescence was quantified immediately in a Synergy H4 hybrid microplate reader (BioTek Instruments Inc., Winooski, VT, USA). Transcriptional potency was converted to fold induction above the corresponding vehicle control value (for AR agonistic potency) or 1.0 × 10^{-9} M DHT (for AR antagonistic potency).

2.5.2. Reporter gene assay for thyroid receptor

Green monkey kidney fibroblast (CV-1) (Chinese Cell Center, Beijing) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% FBS, 100 U/mL penicillin (Sigma) and 100 μg/mL streptomycin (Sigma) in an atmosphere containing 5% CO$_2$ at 37 °C. Cells were seeded in 48-well microplate (5.0 × 10^4 cells per well) in the phenol red free

DMEM medium containing 10% CDS-FBS. Cells were transfected 12 h later with 0.25 μg Gal4 responsive luciferase reporter pUAS-tkluc, 0.1 μg pGal4-L-TR using 2.5 μg Sofast^{TM} transfection reagent per well. After further incubation period of 12 h, the cells were exposed to solvent controls, T_3 and sample extracts for 24 h. Luminescence was quantified immediately in a Synergy H4 hybrid microplate reader (BioTek Instruments Inc., Winooski, VT, USA). Transcriptional potency was converted to fold induction above the corresponding value observed for the vehicle control (for TR agonistic potency) or 5.0×10^{-9} M T_3 (for TR antagonistic potency).

2.6. Data analyses

Agonistic equivalents (EQ) and antagonistic equivalents (AntEQ) for *in vitro* bioassays were calculated using a modified method according to previous studies (Wagner et al., 2013). None of the tested water samples showed AR agonistic or TR agonistic potency, and therefore, only calculation of antagonistic equivalents is shown here. According to previous studies, non-parametric curve fitting, which is most suitable to predict bio-equivalents rather than single-point estimates, was employed (Wagner et al., 2013). The AntEQ was reported as the concentration of flutamide or DnBP divided by the dilution factors of sample that produced an equivalent (20%) depression in the bioassay response to 1.0×10^{-9} mol DHT/L or 5.0×10^{-9} mol T_3/L (Equation (1)). These magnitudes of response could avoid artifacts and biases associated with violation of the assumptions necessary to determine relative potencies of equivalents in extracts of water.

$$\text{AntEQ} = \frac{\text{concentration of known antagonist}}{\text{enrichment factors of tested samples}} \quad (1)$$

Antagonistic equivalents (EQ-IA) derived from concentrations of residues quantified by use of instrumental analysis, were reported as the sum of products of concentrations of individual PAEs multiplied by their respective relative potencies (RePs), which had been determined previously and are shown in Table S2 in supporting information (Hu et al., 2013; Shi et al., 2012).

Concentration-effect curves of individual chemicals or samples did not always have the efficacy (reach the same maximum), or have the same slope, which might have resulted in underestimation or overestimation (Villeneuve et al., 2000). In the present study, 20% equivalent depression and was employed for the mass balance analysis to reduce the uncertainty induced by non-parallelism according to previous studies (Shi et al., 2013b).

All exposures were conducted in triplicate on each plate, and three experiments were performed for each tested sample. Values reported were mean ± standard deviations (SD, n = 3). Triplicate wells were done for each treatment in the bioassays. Data were analyzed by use of one-way ANOVA, followed by Duncan's multiple comparisons test when appropriate using SPSS statistical software (version 11, SPSS Inc., Chicago, Illinois). AR-AntEQs and confidence limits were also calculated using SPSS statistical software based on the above mentioned Equation (1). Curve-fitting analysis was carried out with GraphPad 5.4 (San Diego, CA, USA) using nonlinear regression.

3. Results and discussion

3.1. Cytotoxicity

None of the tested sample extracts from Tai Lake affected viability or proliferation of CV-1 cells alone or in the presence of T_3. Cytotoxic responses of CV-1 were observed for extracts from HR-1 and HR-3 at the maximal tested concentration (200 times the original concentration in the raw water), resulting in decrease expression as 62% and 79% of control. However, all of samples from the Yangtze River exhibited cytotoxic effects for CV-1 at 100 equivalents (100-fold concentrated from original waters) (YR-1, YR-3 and YR-4) or 50 equivalents (YR-2) (Supporting Information, Fig. S1 and Fig. S2). Similar results were obtained for MDA-kb2. All extracts of water from the Yangtze River caused a decrease of cell viability at 200 and 100 equivalents, and sample from YR-2 was more cytotoxic and toxicity was observed for samples of 25 equivalents. Generally, the two cell lines showed similar responses towards organic extracts of surface waters. These results indicate that more cytotoxic compounds existed in water of the Yangtze River. This is consistent with results, which showed that water from the Yangtze River could induce cytotoxicity on rat gonad cells (Wang et al., 2010). Moreover, cytotoxicity has been observed in effluents from petrochemical plants discharged to the Yangtze River (Floehr et al., 2013). None of the blank sample exhibited cytotoxicity.

3.2. Assay validation

The natural ligand of the AR, DHT induced luciferase potency in a concentration-dependent manner in the MDA-kb2 reporter gene assays based on a high throughput 384-well microplate format. DHT induced luciferase potency in the range of 1.0×10^{-11} M to 1.0×10^{-7} M, with maximal induction of 12.4-fold relative to that of the vehicle control achieved at a concentration of 1.0×10^{-7} mol DHT/L (Supporting Information, Fig. S3). The known AR antagonist, flutamide, caused a typical concentration-effect curve in the presence of 1.0×10^{-9} M DHT with an EC_{20} of 6.8×10^{-7} M (Supporting Information, Fig. S4). Reliability and sensitivity of the CV-1 cell line was also assessed in this study. The standard chemical T_3 induced luciferase potency in a concentration-dependent manner in the range of 1.0×10^{-11} to 1.0×10^{-6} M (Supporting Information, Fig. S5). The known TR antagonist, DnBP, caused inhibition of the luciferase potency induced by 5.0×10^{-9} mol T_3/L in a concentration-dependent manner (Supporting Information, Fig. S6). No significant induction of luciferase was observed in any of the solvent controls.

3.3. Androgenic receptor and thyroid receptor agonist and antagonist potencies

None of the samples of water or blanks exhibited AR agonistic potency or TR agonistic potency. Similar results have been observed in previous studies, which indicated that the aquatic environment in Taiwan showed little TR agonist potency (Chen et al., 2014). Municipal waste water in China that was studied by Fang et al. exhibited no androgenic potency either (Fang et al., 2012). Results of the studies which are reported here indicated that agonists of the TR or AR in samples from the environment were limited (Sun et al., 2012). Results of previous studies have indicated that mixtures have a potential antagonizing effect when some AR or TR agonists coexist (Ghisari and Bonefeld-Jorgensen, 2009), which could also contribute to the undetectable agonists.

All twelve water samples exhibited TR antagonistic potencies in a concentration-dependent manner (Fig. 2). No measurable potency was observed in blank samples. Extracts significantly suppressed expression of reporter gene to less than 30% of the potency induced by 1.0×10^{-9} M T_3, at the maximal concentration (200 equivalents) in water from all sites. Thyroid receptor antagonistic equivalents (TR-AntEQs) for the twelve samples of water ranged from 3.6 to 76.1 μg DnBP/L (Table 1). The lowest activity were detected in extracts of samples of water from Tai Lake at sites TL-1, TL-3 and TL-4, which indicated that in general concentrations of TR-AntEQs were less than those at other sites and that water was relatively clean at these sites. The greatest TR antagonistic potency was measured for samples from sites YR-2 and YR-3, both of which were from the Yangtze River Delta. YR-2 is in TZ city, which
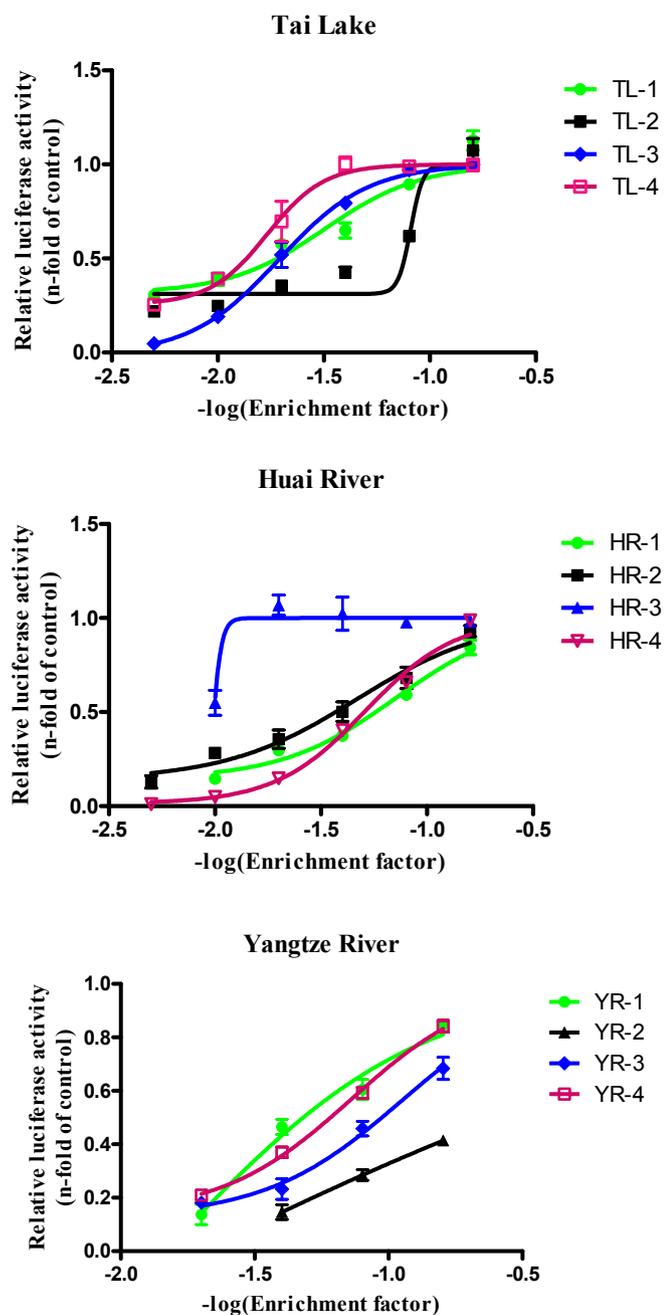
Tai Lake

Huai River

Yangtze River

Fig. 2. Concentration-dependent thyroid receptor (TR) antagonist potencies in extracts of water as measured by the CV-1 cell line based reporter gene assay. Extracts of water in cell culture media were tested at 6.25, 12.5, 25, 50, 100 and 200 times of the original concentration in water samples. Cells were exposed to extracts in parallel with 5 nM T_3. The TR antagonist potency was expressed as relative expression versus the untreated cells (control) (mean ± SD).

Table 1
Thyroid receptor antagonist equivalents (TR-AntEQ, μg DnBP/L) and androgen receptor antagonist equivalents (AR-AntEQ, μg flutamide/L) derived from reporter gene assays for the water samples.

Locations	Thyroid receptor (μg DnBP/L)			Androgen receptor (μg flutamide/L)		
	TR-AntEQs	95% confidence limits		AR-AntEQs	95% confidence limits	
		Lower	Upper		Lower	Upper
TL-1	8.1	5.0	19.2	16.2	9.8	44.2
TL-2	20.4	8.2	17.2	14.1	12.2	16.6
TL-3	6.3	5.5	7.3	15.0	11.3	23.3
TL-4	3.6	2.6	6.3	59.1	43.7	89.7
HR-1	25.0	15.4	60.4	13.8	9.9	24.1
HR-2	18.6	11.5	43.1	25.6	15.7	8931.3
HR-3	14.9	9.6	33.3	2.3	2.1	2.6
HR-4	15.6	11.4	25.7	88.9	61.4	30.0
YR-1	20.1	14.5	34.6	242.6	116.4	31.1
YR-2	76.1	39.3	62.6	105.2	50.8	3061.3
YR-3	38.9	23.2	111.5	49.8	39.1	69.5
YR-4	21.9	15.5	39.4	31.6	22.8	60.0

the aquatic environment in Taiwan and Beijing (Chen et al., 2014), and effluent from waste-water treatment plant was suggested to be a potential source of TR disrupting compounds (Li et al., 2011).

Results of a previous study have indicated that DnBP might induce T_3-dependent activation of the TRβ gene in metamorphosing tadpoles at a concentration of 1.1×10^3 μg/L (Sugiyama et al., 2005). TR-AntEQs of grade II surface waters measured during the present study were less than that concentration. However, resent study has indicated that the lowest observable effect concentration (LOEC) for DnBP to influence concentrations of sex steroid hormones and gonadal development in juvenile Murray rainbow fish, which is 5 μg/L (Bhatia et al., 2014). All detected TR-AntEQs in extracts of grade II surface waters were higher than this level except location TL-4. Moreover, these integrated equivalents were significantly greater than the national criteria of DnBP for water sources in China, which is 3 μg/L and DnBP is not listed in the criteria for surface water in China. Thyroid hormone disrupting potentials warranted further attention.

All twelve water samples exhibited AR antagonistic potencies in a concentration-dependent manner (Fig. 3). No potency was observed in blank samples. AR antagonist potencies ranged from 2.3 to 242.6 μg flutamide/L (Table 1). This is slightly less than concentrations of equivalents detected in surface water form the Pearl River, which were 20.4–935 μg flutamide/L (Zhao et al., 2011). The greatest potency was observed in samples of water from site YR-1 (242 μg flutamide/L), which was greater than the LOEC of flutamide to induce testis-ova in male medaka (200 μg/L) (Kang et al., 2006; Leon et al., 2007). This result also indicates a potential risk to aquatic biota and human health, which warrants further attention. Site YR-1 from NJ city is near the place where many passenger ships and docks occur, and the industrial waste water might be the main pollution sources. Concentrations of AR-AntEQ of extracts of water from site YR-2 on the Yangtze River was also relatively great and more attentions should be paid to this location. In general, the AR antagonistic potencies in the Yangtze River watershed were also great and the pollution is highlighted. These results are consistent with those of previous studies, which demonstrated that water samples from the Yangtze River had actual toxicity to reproductive systems of male mice (Zhao et al., 2009).

Extracts of water elicited both TR antagonist and AR antagonist potencies, simultaneously (Fig. 4). Potencies of AR and TR antagonists of samples of water from different river basins were significantly different. Extracts of water from Tai Lake and upper reaches of the Huai River showed lesser potencies. These results are
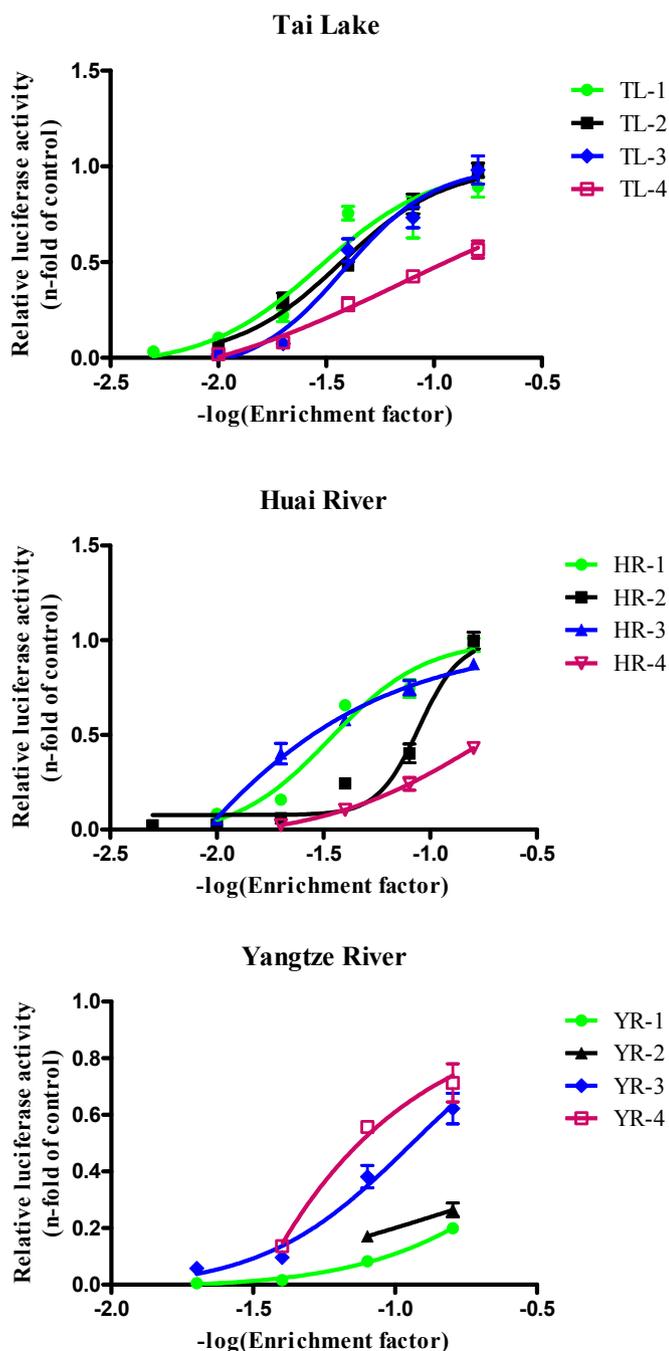
has many chemical industry parks along the river. YR-3 is in NT city and domestic sewage might be the main pollution source. Potencies of extracts of water from farther downstream did not appear to be greater which might be caused by the complex conditions and the large amount of water in the lower reaches of the Yangtze River. TR-AntEQs for samples from the Huai and Yangtze Rivers were greater than previously reported equivalents in source water samples being less than 8.0 μg DnBP/L during the wet season in 2010 (Shi et al., 2012). This might be due to effluents from industrial plants and farm lands in the vicinity of these grade II surface waters. Strong TR antagonist potency was also detectable in

Tai Lake

Huai River

Yangtze River

Fig. 3. Concentration-dependent androgen receptor (AR) antagonist potencies in extracts of water as measured by the MDA-kb2 cell line AR reporter gene assay. Extracts of water in cell culture media were tested at 6.25, 12.5, 25, 50, 100 and 200 times of the original concentration in water samples. Cells were exposed to extracts in parallel with 1 nM DHT. The AR antagonist potency was expressed as relative expression versus the untreated cells (control) (mean ± SD).

consistent with those observed for the cytotoxicity test. Extracts of water from the Yangtze River showed greater potencies for both TR and AR, which indicated that the river was contaminated with TR and AR agonists. Locations for which potencies of TR antagonists were greatest, also exhibited greater AR antagonists. This indicated that contaminants in extracts could induce both TR and AR antagonist. Results of previous studies indicated that most of the TR antagonists might also induce AR antagonist potencies and is in accordance with the present results (Shen et al., 2009). Conse-

quently, the identification of potential ligand and further environmental monitoring are essential.

3.4. Potency balance analysis

PAEs were detectable in all the locations at concentrations ranging from 6.19 to 205.4 μg/L. DEHP, DiBP and DnBP were the major PAEs observed, accounting for more than half of total PAEs observed. Concentrations of DnBP and DEHP in waters ranged from 2.17 to 67.75 and 0.08–3.31 μg/L, respectively. Concentrations of DEHP were less than the national standard for drinking water sources (8.0 μg/L) in China. Detectable concentrations of DnBP, which were greater than the national standard (3.0 μg/L) in all samples except HR-3. These results indicated that pollution of surface waters with DnBP was serious and more measures should be taken for its mitigation. Concentrations of DnBP and DEHP were comparable with the results detected in raw drinking water in the Yangtze River (as great as 6.2 μg/L) and Southern California (8.3–5.9 μg/L) (Loraine and Pettigrove, 2006; Wu et al., 2009). Concentrations of DiBP ranged from 2.81 to 132.49 μg/L. Although, in China, there is no water quality standard for DiBP, the relatively large concentrations observed cannot be ignored. Greatest concentrations of DnBP, DiBP and DEHP were found at site YR-2, where great TR and AR antagonistic potencies were found.

Predicted AR and TR antagonistic potencies were also calculated based on concentrations of PAEs and their individual RePs (Table 2). Concentrations of TR antagonistic equivalents based on instrumental analysis (TR-EQ-IA) in water ranged from 2.36 to 73.7 μg DnBP/L. The greatest concentration and strongest TR antagonist potency, was observed for DnBP, which accounted for over 80% of the TR-EQ-IA, which is in agreement with the results of a previous study conducted in Beijing (Li et al., 2010). When the TR-AntEQs derived from the bioassay were compared with TR-EQ-IA calculated from the concentrations of phthalates, 79%–100% of the total TR-EQ-IA were contributed by PAEs in all the water samples except HR-3 (Fig. 5). It could be speculated that PAEs, especially for DnBP might be the major TR antagonist potency in most of the grade II surface water, while other contaminants might contribute to the equivalents at HR-3. This is in accordance with our previous studies considering about the endocrine disrupting potencies in water sources, which indicated that PAEs might contribute to the TR antagonist potencies (Shi et al., 2012). Concentrations of AR antagonistic equivalents based on instrumental analysis (AR-EQ-IA) in water ranged from 2.3 to 242.6 μg flutamide/L. DiBP accounted for over 60% of the AR-EQ-IA. However, for half of the sites, PAEs contributed to less than 50% of the AR-AntEQs. This result disagrees with our previous results showing that PAEs is responsible for the AR antagonistic potencies in the source water. Results indicated that, when compared with the source water, more AR antagonists might exist in the grade II surface water. Previous researchers have
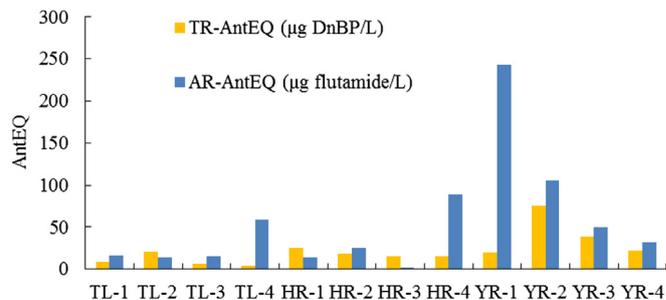

Fig. 4. Correlations between potencies of thyroid receptor (TR) antagonist and androgen receptor (AR) antagonist.

Table 2
Concentrations of phthalate esters, thyroid receptor (TR) antagonistic equivalents based on instrumental analysis (TR-EQ-IA) and androgen receptor (AR) antagonistic equivalents based on instrumental analysis (AR-EQ-IA) in water (μg/L).

	DMP	DEP	DiBP	DnBP	BBP	DEHP	TR-EQ-IA	AR-EQ-IA
TL-1	0.11	0.01	16.27	7.64	0.01	0.11	8.38	11.24
TL-2	0.11	0.03	2.89	13.75	1.28	0.59	13.91	2.69
TL-3	0.03	0.02	10.51	5.39	0.28	0.22	5.88	7.29
TL-4	0.01	0.01	6.16	3.20	0.10	0.16	3.50	4.28
HR-1	0.19	0.92	2.81	21.98	2.16	1.52	22.40	3.14
HR-2	0.13	0.25	20.49	14.13	0.24	0.83	15.19	14.41
HR-3	0.04	0.02	3.86	2.17	0.02	0.08	2.36	2.69
HR-4	0.02	0.15	21.05	11.82	0.11	0.84	12.80	14.66
YR-1	0.28	0.18	34.22	16.77	0.79	0.70	18.38	23.72
YR-2	0.17	0.76	132.94	67.45	0.76	3.31	73.73	92.17
YR-3	0.03	0.10	73.41	27.33	0.87	1.03	30.72	50.38
YR-4	0.64	0.05	23.68	17.31	0.06	0.44	18.42	16.70

shown that when some agonists coexist, mixtures have a potential antagonizing effect (Ghisari and Bonefeld-Jorgensen, 2009). These might be the reasons that PAEs contributed less to the equivalents based on instrumental analysis in this study. Further study was needed to examine the synergism or retard potencies of these endocrine disrupting chemicals.

Qualitative analysis was further conducted to identify the responsible chemicals. NP, Octylphenol (OP), Methyl Parathion, Endosulfan, BPA, Benzo[a]pyrene, parathion and chlorpyrifos were detectable in most of the extracts. Concentrations of BPA in HR-3 was greater than the other sites and could be the major contributor for TR-AntEQs at this site. Some chemicals, including OP, NP, chlorpyrifos and BPA have been reported as strong AR antagonists

(Ghisari and Bonefeld-Jorgensen, 2009; Shen et al., 2009). Frequencies of detection of these chemicals were great, and frequencies of detection of OP, NP and Benzo[a]pyrene were more than 95%. Concentrations of benzo[a]pyrene in water samples from the Yangtze River were greater than those at other sites, where greatest cytotoxicity were found. Potencies observed in extracts might be due to these chemicals especially benzo[a]pyrene, so additional quantitative analyses are needed.

Grade II surface water can be used to supply drinking water. It has been reported that the removal efficiency of PAE in drinking water treatment processes which include the activated carbon filtration was effective (Li et al., 2010). However, treatment of water in most drinking water treatment plants does not include use of activated carbon filtration and limited proportions of other EDCs could be removed (Luo et al., 2014; Padhye et al., 2014). This illustrated that potential risks might be posed to residents if these grade II surface waters are used as sources of drinking. More effective efforts should be taken before the choosing of source water. Further research should be considered to assess the fate as well as status and trends in concentrations of these compounds.

Results of the present study showed that potencies of mixtures of AR and TR antagonists were ubiquitous in grade II surface water in East China. Samples of water from the Yangtze River showed the greatest potency for AR, TR and cytotoxicity. TR-AntEQ ranged from 3.6 to 76.1 μg dibutyl phthalate (DnBP)/L and is mainly contributed by DnBP and DiBP. AR-AntEQ ranged from 2.3 to 242.6 μg flutamide/L, and PAEs were not the main contributor at most of the locations. The frequently of detection for OP, NP and benzo[a]pyrene might be responsible. Further identification of potential candidates for AR antagonists in the grade II surface water should be undertaken to better protect the aquatic environment.
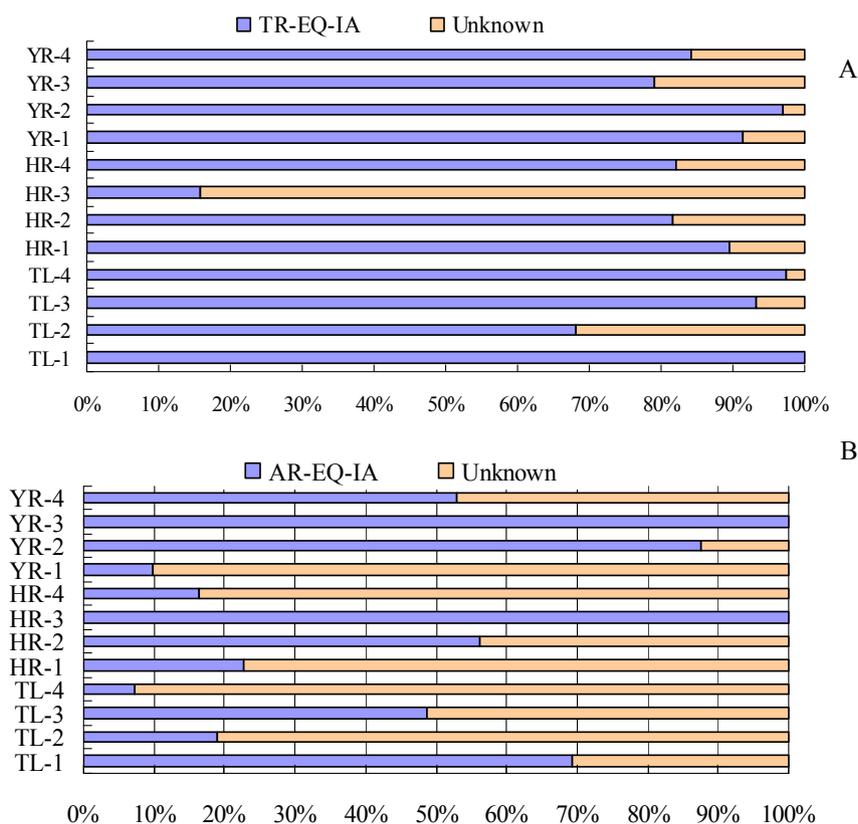

Fig. 5. Contributions of phthalates to the (A) thyroid receptor antagonist equivalents (TR-AntEQ) and (B) androgen receptor antagonist equivalents (AR-AntEQ) in water.

Acknowledgment

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2015.09.018.

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Causes of Endocrine Disrupting Potencies in Surface Water in East China

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Supporting Information, Table S2. Recoveries and limits of quantification (LOQ) for analytes.

| Chemicals	CAS	LOQ (ng/L)	Procedural recovery (n=3)		Matrix spike recovery (n=3)	
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Phthalate esters	Dibutyl phthalate (DnBP)	84-74-2	2.0	102	2.3	110	3.5
	Diisobutyl phthalate (DiBP)	84-69-5	0.30	91	5.2	98	5.0
	Benzyl butyl phthalate (BBP)	85-68-7	1.0	96	3.1	102	3.3
	Diethyl phthalate (DEP)	84-66-2	1.0	82	5.5	82	3.7
	Dimethyl phthalate (DMP)	131-11-3	1.0	93	4.2	94	4.3
	Di-2-ethylhexyl phthalate (DEHP)	117-81-7	0.30	99	2.0	107	3.1

LOQ: Limits of quantitation (S/N =10).

RSD: Relative standard deviation.

Supporting Information, Table S2 Thyroid receptor antagonist relative potencies (TR-Ant-RePs, µg DnBP/L) and androgen receptor antagonist relative potencies (AR-Ant-RePs, µg flutamide/L) for phthalate esters.

|	TR-Ant-RePs (µg DnBP/L)	AR-Ant-RePs (µg flutamide/L)
DMP	0.006	0.025
DEP	0.017	0.049
DiBP	0.045	0.666
DnBP	1.000	0.052
BBP	0.002	0.034
DEHP	0.015	0.007

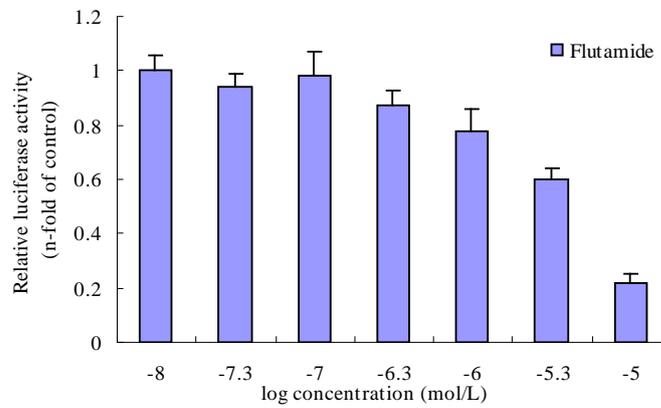Supporting Information, Figure S1 Cytotoxicity of water extracts for MDA-kb2
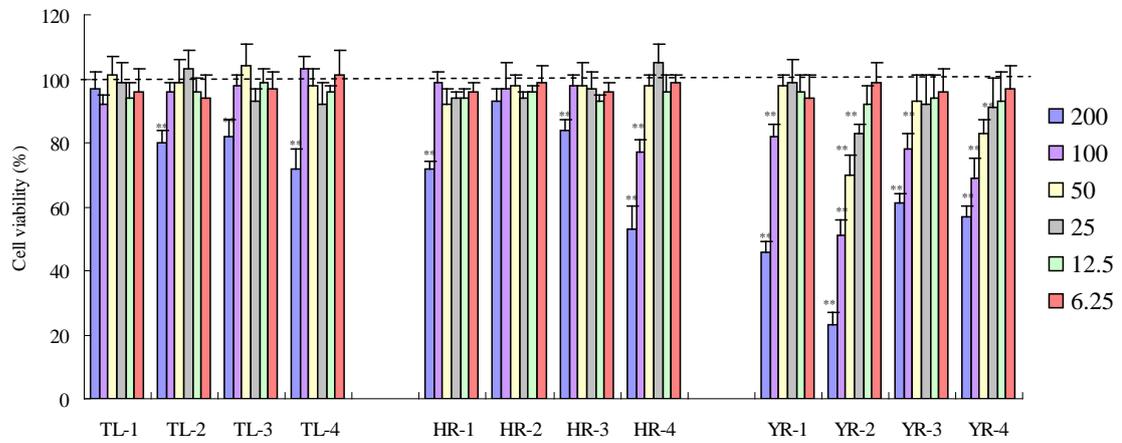
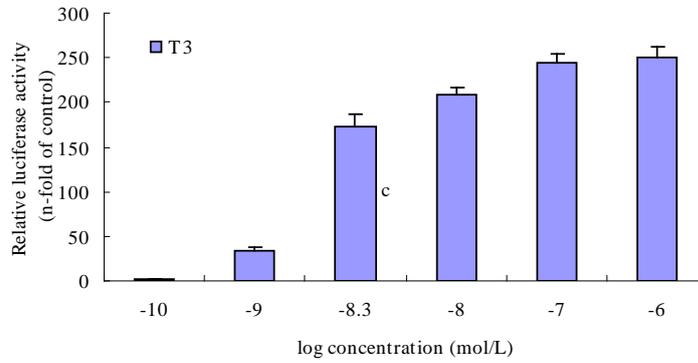Supporting Information, Figure S2 Cytotoxicity of water extracts for r CV-1
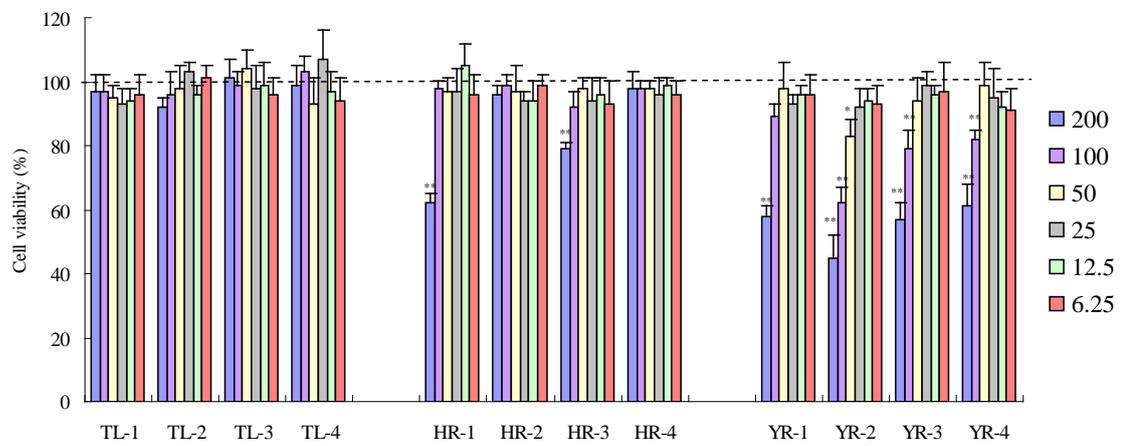
Supporting Information, Figure S3. The relative luciferase activity induced by DHT in reporter gene assay based on MDA-kb2 cell lines.
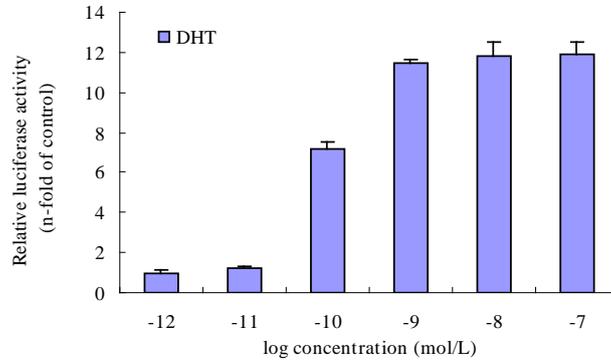
Supporting Information, Figure S4. The relative luciferase activity comparing to 1.0×10^{-9} mol DHT/L treated with flutamide in reporter gene assay based on MDA-kb2 cell lines.

Supporting Information, Figure S5. The relative luciferase activity induced by T_3 in reporter gene assay based on CV-1 cell lines.

Supporting Information, Figure S6. The relative luciferase activity comparing to 5.0×10^{-9} mol T_3/L treated with DnBP in reporter gene assay based on CV-1 cell lines.