Journal of Hazardous Materials 182 (2010) 494–502

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Effects of sulfathiazole, oxytetracycline and chlortetracycline on steroidogenesis in the human adrenocarcinoma (H295R) cell line and freshwater fish *Oryzias latipes*

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ARTICLE INFO

Article history:
Received 2 November 2009
Received in revised form 13 June 2010
Accepted 14 June 2010
Available online 19 June 2010

Keywords:
Antibiotic
Aromatase
Endocrine disruption
Pharmaceutical
Steroidogenesis

ABSTRACT

Pharmaceuticals in the environment are of growing concern for their potential consequences on human and ecosystem health. Alterations in the endocrine system in humans or wildlife are of special interest because these alterations could eventually lead to changes in reproductive fitness. Using the H295R cell line, the potential endocrine disrupting effects of six pharmaceuticals including diclofenac, erythromycin, sulfamethazine, sulfathiazole, oxytetracycline, and chlortetracycline were investigated. After exposure to each target pharmaceutical for 48 h, production of 17β-estradiol (E2) and testosterone (T), aromatase (CYP19) enzyme activity, or expression of steroidogenic genes were measured. Concentrations of E2 in blood plasma were determined in male Japanese medaka fish after 14 d exposure to sulfathiazole, oxytetracycline, or chlortetracycline. Among the pharmaceuticals studied, sulfathiazole, oxytetracycline and chlortetracycline all significantly affected E2 production by H295R cells. This mechanism of the effect was enhanced aromatase activity and up-regulation of mRNAs for *CYP17*, *CYP19*, and *3βHSD*, all of which are important components of steroidogenic pathways. Sulfathiazole was the most potent compound affecting steroidogenesis in H295R cells, followed by chlortetracycline and oxytetracycline. Sulfathiazole significantly increased aromatase activity at 0.2 mg/l. In medaka fish, concentrations of E2 in plasma increased significantly during 14-d exposure to 50 or 500 mg/l sulfathiazole, or 40 mg/l chlortetracycline. Based on the results of this study, certain pharmaceuticals could affect steroidogenic pathway and alter sex hormone balance. Concentrations of the pharmaceuticals studied that have been reported to occur in rivers of Korea are much less than the thresholds for effects on the endpoints studied here. Thus, it is unlikely that these pharmaceuticals are causing adverse effects on fish in those rivers.

1. Introduction

Pharmaceuticals are used in both human and veterinary medicine, to treat and prevent diseases, and to promote growth of some livestock [1]. Widespread use of pharmaceuticals eventually leads to the contamination of surface waters environment [2]. Approximately 80–100 pharmaceuticals and their metabolites have been detected in sewage, surface water, groundwater, and drinking water worldwide [3–10].

Although pharmaceuticals are designed for specific physiological functions, they could cause unintended adverse effects on non-target organisms even at relatively small concentrations [11]. However, toxicological studies on pharmaceuticals in the environment are mostly limited to the lethal effects during acute exposures. There are information gaps that need to be filled in order to fully understand the consequences of pharmaceutical contamination in the environment and to develop appropriate

Abbreviations: CYP19, aromatase; E2, 17β-estradiol; ELISA, enzyme-linked immunosorbent assay; DMSO, dimethyl sulfoxide; H295R, human adrenocarcinoma; T, testosterone; E1, estrone.

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doi:10.1016/j.jhazmat.2010.06.059

management plans. One such gap is effects on during chronic, sublethal exposures and potential mechanisms of effect. Effects of pharmaceuticals on steroidogenesis of non-target aquatic organisms are of interest because such disruption of endocrine function could result in effects on fecundity [12].

Because estrogenic effects of pharmaceuticals has been observed *in vitro*, which suggests the need for further *in vivo* studies on the estrogenic potential of environmental pharmaceuticals. Using a recombinant yeast system expressing human estrogen receptor α, six pharmaceuticals including cimetidine, fenofibrate, furosemide, paracetamol, phenazone and tomoxifen out of 37 pharmaceuticals tested, were found to be estrogenic [12]. Based on the E-screen assay, 11 pharmaceuticals including atenolol, erythromycin, gemfibrozil, and paracetamol of 14 that were tested were found to have the potential to interfere with the endocrine system [13]. Antibiotics, such as amoxicillin, tylosin, and oxytetracycline, can modulate gene expression and hormone production related to steroidogenesis [14]. In male Japanese medaka fish (*Oryzias latipes*), oxytetracycline and chlortetracycline induced production of vitellogenin [15,16]. There is still a lack of understanding of the mechanisms by which such alterations could be manifested. Information on the mechanisms of such effects is necessary to be able to aggregate chemicals into groups of similar mechanisms so that assessments of the mixtures can be made. In addition to acting through hormone receptor-mediated pathways, environmental chemicals can alter endocrine function by modulating production or breakdown of steroid hormones [17,18].

We chose diclofenac, erythromycin, sulfamethazine, sulfathiazole, oxytetracycline, and chlortetracycline as model chemicals based on the frequency of detection in Korean waterways [19,20], and tested them for their effects on steroidogenesis *in vitro* with human adrenocarcinoma (H295R) cells and *in vivo* with male medaka fish.

The H295R steroidogenesis assay was developed for the quantitative evaluation of xenobiotic effects on transcription of genes involved in steroidogenesis [17,18,21]. H295R cells express major key enzymes involved in the synthesis of steroid hormones, and the assay has been successfully used for the characterization of effects of chemicals on steroidogenesis. For compounds that were determined to affect steroid hormone production (testosterone (T) and 17β-estradiol (E2)), the mechanisms of steroidogenic effect were investigated by measuring changes in aromatase (CYP19) enzyme activity and expression of genes (*3βHSD2*, *CYP11β2*, *CYP17*, *CYP19*, and *17βHSD*) in the steroidogenic pathways in H295R cells. Fish were exposed to target pharmaceuticals and concentrations of E2 in blood plasma (plasma).

2. Materials and methods

2.1. Test chemicals

Test pharmaceuticals included diclofenac sodium salt (CAS No. 15307-79-6), erythromycin (CAS No. 114-07-8), sulfamethazine (CAS No. 1981-58-4), sulfathiazole sodium salt (CAS No. 144-74-1), oxytetracycline hydrochloride (CAS No. 2058-46-0), and chlortetracycline hydrochloride (CAS No. 57-62-5). All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). Each compound was dissolved in dimethyl sulfoxide (DMSO) and the final DMSO concentration in the exposure medium was less than 0.1% (v/v).

2.2. H295R cell culture

H295R cells (CRL-2128) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37 °C in a 5% CO_2 atmosphere. H295R cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 Nutrient mixture (DMEM/F12) (Sigma D-2906; Sigma–Aldrich) supplemented with 1% ITS + Premix (BD Biosciences, San Jose, CA, USA), 2.5% Nu-Serum (BD Biosciences), and 1.2 g/l Na_2CO_3 (Sigma–Aldrich). Medium was changed every 4 d, and cell subculture was performed every 7 d.

2.3. Hormone measurement of H295R cell

T and E2 were measured following methods described by Hecker et al. [21] and He et al. [22]. H295R cells were seeded into 24-well plates at a concentration of 3×10^5 cells/ml in 1 ml of medium per well. After 24 h, cells were exposed to pharmaceuticals at concentrations ranging from 0.02 to 20 mg/l for 48 h. Cells were inspected under a microscope for viability and cell number. In instances where exposure resulted in cell viability less than 85%, the cells were not used for assays that evaluated hormone production, aromatase activity and gene expression [14,23]. The culture medium was collected and kept frozen at −80 °C. Frozen medium was thawed on ice, and 500 μl medium was extracted twice with 2.5 ml diethyl ether. The solvent phase containing target hormones was evaporated under a stream of nitrogen, and the residue was reconstituted in 300 μl enzyme-linked immunosorbent assay (ELISA) buffer (Cayman Chemical, Ann Arbor, MI, USA) and frozen at −80 °C for subsequent analysis. Medium extracts were diluted 1:75 and 1:1 for T and E2, respectively. Hormones were measured by competitive ELISA following the manufacturer's recommendations (Cayman Chemical; Testosterone [Cat # 582701], 17β-Estradiol [Cat # 582251]).

2.4. Aromatase activity assay of H295R cell

Aromatase enzyme activity was measured by use of the method described by He et al. [22] with some modifications. Direct and indirect effects on aromatase activity were evaluated for pharmaceuticals that caused significant changes in hormone production [23]. To measure direct effects of chemicals on aromatase activity, cells were treated with a range of concentrations (0.02–2 mg/l) of each target pharmaceutical in the medium that contained 54 nM 1β-^{3}[H]-androstenedione (PerkinElmer, Boston, MA, USA) with no pre-exposure to a target pharmaceutical. In order to measure indirect effects of chemicals on aromatase activity, H295R cells were initially exposed to various concentrations of each pharmaceutical for 48 h, after which the cells were washed twice and incubated with 0.25 ml of serum-free medium that contained 54 nM 1β-^{3}[H]-androstenedione. After 1.5 h incubation at 37 °C and 5% CO_2, the cells were placed on ice to stop the reaction. A 200 μl aliquot of medium was removed and added to chloroform and dextran-coated charcoal to remove all remaining 1β-^{3}[H]-androstenedione. Aromatase activity was determined by the rate of conversion of 1β-^{3}[H]-androstenedione to estrone. The quantity of ^{3}H in extracts of medium was determined by liquid scintillation counter (Beckmann LS6500, Beckmann Coulter Inc., Fullerton, CA, USA). Forskolin (0.1, 1, and 10 μM) was used as a positive control for aromatase induction, while prochloraz (0.1, 1, and 10 μM) was used as a negative control for aromatase catalytic inhibition.

2.5. Quantitative PCR assay of H295R cell

Transcription of mRNA of five steroidogenic genes plus one housekeeping gene (*β-actin*) were measured in cells exposed to pharmaceuticals that caused significant changes in hormone production by the method outlined by Hilscherova et al. [17]. Briefly, 1 ml cell suspension (initial cell density of 3×10^5 cells/ml) was transferred to each well of a 24-well culture plate. After 24 h, cells were exposed to chemicals for another 48 h, and then total RNA