Journal of Hazardous Materials xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Persistent endocrine disruption effects in medaka fish with early life-stage exposure to a triazole-containing aromatase inhibitor (letrozole)

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HIGHLIGHTS

- We assess ecotoxicological impact of a clinic drug, letrozole (LET) in aquifers.
- We show reproduction toxicity in medaka with early life stage exposure to LET.
- LET causes endocrine disruption effects in adulthood and progeny of medaka.
- LET exposure interrupts gene expression for controlling hormone balance in medaka.
- We suggest toxicity evaluation methods for aromatase inhibitory drugs using medaka.

ARTICLE INFO

Article history:
Received 16 October 2013
Received in revised form 28 January 2014
Accepted 11 February 2014
Available online xxx

Keywords:
Letrozole
Medaka (*Oryzias latipes*)
Cytochrome P450 (CYP)
Aromatase inhibitor

ABSTRACT

Letrozole (LET) is a triazole-containing drug that can inhibit the activity of cytochrome P450 aromatase. It is an environmentally emerging pollutant because of its broad use in medicine and frequent occurrence in aquifers receiving the effluent of municipal or hospital wastewater. However, the toxic impact of LET on fish populations remains unclear. We exposed medaka fish (*Oryzias latipes*) at an early stage of sexual development to a continuous chronic LET at environmentally relevant concentrations and assessed the endocrine disruption effects in adulthood and the next generation. LET exposure at an early life stage persistently altered phenotypic sex development and reproduction in adults and skewed the sex ratio in progeny. As well, LET exposure led to a gender-different endocrine disruption as seen by the interruption in gene expression responsible for estrogen synthesis and metabolism and fish reproduction. LET interfering with the aromatase system in early life stages of medaka can disrupt hormone homeostasis and reproduction. This potent aromatase inhibitor has potential ecotoxicological impact on fish populations in aquatic environments.

1. Introduction

As technology of analytical chemistry rapidly develops, various anthropogenic organic contaminants, including pesticides, pharmaceuticals and personal care products (PPCPs) are frequently detected at nanogram to microgram per liter levels in aquifers worldwide. Such aquifers include wastewater treatment work effluent, surface and ground water, and even drinking water supplies [1–4]. PPCPs include a large and diverse group of medicinal compounds used for the diagnosis, cure, mitigation, treatment, or prevention of diseases in humans and animals. The main routes of PPCPs entering the environment are manufacturing, hospital or domestic effluent, whereby the pharmaceutical (as the parent compound or as metabolites) from treated patients is directly released into the wastewater system or via agricultural runoff and irrigation return waters (pesticides and animal husbandry hormones and medicines) [3,4]. Many PPCPs of emerging concern are potential endocrine disruptors, which at minute levels are sufficient to cause reproductive and developmental dysfunction in wildlife and numerous species and may pose a significant threat to human health [5,6].

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http://dx.doi.org/10.1016/j.jhazmat.2014.02.013
0304-3894/

Endocrine disruptors interfere with hormone-signaling by several modes of action including (1) competitively binding to the estrogen or androgen receptor (ER or AR, respectively), thus activating the transcription of sex-hormone-dependent genes (e.g., xenoestrogens or xenoandrogens); (2) binding but not activating the ER or AR (e.g., anti-estrogens or anti-androgens); (3) modifying enzymatic pathways involved in biosynthesis of sex hormones (e.g., an aromatase inhibitor [AI], which blocks the synthesis of 17β-estradiol [E2] from testosterone [T]); and (4) other unclear mechanisms [7,8]. Numerous screening tests have assessed the effects of estrogen mimics [7], but much less research effort has focused on other types of endocrine disruptors, and the associated ecotoxicology is limited.

Conazoles are a class of imidazole- or triazole-containing drugs that have been commonly used as fungicides in agriculture and medicine and as non-steroidal antiestrogens for treating human disease [9]. This broad use of azoles is based on their inhibition of certain pathways of steroidogenesis by binding to the enzymes sterol 14-alpha-demethylase (CYP51) or cytochrome P450 aromatase (CYP19). However, the specificity of the enzyme inhibition of several azoles is poor, with evidence of adverse health effects on carcinogenesis via CYP (e.g., CYP1A, 2B, 3A etc.)-mediated metabolism in mammalians and other non-targeted organisms [10–13]. Several conazoles inhibit steriodogenic CYPs (e.g., CYP19, CYP11A and CYP17) in mature fish and depress circulating sex steroid concentrations, thereby decreasing hepatic production and ovarian deposition of vitellogenin (VTG; egg yolk protein) and reducing egg production [14–16]. Indeed, many azole compounds occur ubiquitously in the aquatic environment, and the public has raised concerns about their environmental safety [6,17,18].

Azoles of emerging concern include letrozole (Femara, LET), a triazole derivative, most widely used as a potent AI for first-line therapy of hormonally responsive breast cancer or in assisted reproduction [19,20]. LET is a moderately hydrophobic compound (Log p = 3.385) that may easily be excreted in human urine and transported through aquifers after sewage discharge. LET has been frequently detected in municipal sewage, hospital effluent or the receiving river water [21]. LET was found to inhibit oocyte growth and reduce plasma vitellogenin (VTG) levels in female medaka adults [22]. A prototype AI, fadrozole, at 51.7 μg/L, significantly induced testis growth in fathead minnows [23] and retarded oocyte maturation in female coho salmon during vitellogenesis [24]. As well, exposure of juvenile zebrafish to fadrozole (10–100 μg/L) during early development inhibited differentiation and development of female gonads [25]. However, little attention has been paid to the endocrine-disrupting effect and its association with CYP-mediated mechanisms of environmental LET on young fish populations, especially during early development, the sensitive window of sex determination and development. Medaka (*Oryzias latipes*) is a gonochorist fish with genetic sex (a XY–XX sex-determined system) determined at fertilization that does not change with temperature or hormones. The window of sex differentiation and development starts from 1 to 2 days before hatching to 40 days after hatching and then fish retain the same phenotypic sex throughout life [26]. As well, a unique strain such as female leukophore-free (FLFII) medaka, carrying multiple DNA markers of genotypic sex, is a superior model organism for studies of endocrine disruptors [27].

Here, we treated FLFII medaka at an early sex-development stage with environmentally relevant concentrations of LET and assessed the latent endocrine-disruption effects in adults (F0) and transgenerational effects in F1 progeny. The endpoints included hatchability and survival of embryos and larvae, phenotypic sex characteristics (indices of secondary sex characters and gonadal histopathology), gene expression in liver and gonads, hepatic CYP activity and the reproductive performance in F0 adults as well as F1 sex ratio.

2. Materials and methods

2.1. Fish and fish care

The FLFII medaka was a kind gift from the National Institute for Basic Biology, Japan. The brood stock were maintained and bred at the Department of Agricultural Chemistry of National Taiwan University (NTU) according to the protocols of the NTU Institutional Animal Care and Use Committee. Briefly, fish were reared in dechlorinated tap water with stable water quality under rigorous environmental conditions with a consistent temperature (26 ± 1 °C) and a photoperiod of 14:10 h (light:dark).

2.2. Experimental design

Egg clutches spawned from 1-day spawn of the same stock of females were separated and disinfected in a H_2O_2 solution (0.9%) for 5 min. Stocks of LET and 17α-ethinylestradiol (EE2) were prepared in acetone and diluted with embryo-rearing medium (ERM) [28] for fish exposure. The synthetic estrogen EE2 was used to confirm the effects of estrogen mimics on our FLFII medaka and exposure systems, as well as for comparisons with LET-induced effects. Approximately 400 embryos at stage 10 were randomly divided into 4 groups (100 each) and treated with LET (50 and 500 μg/L), EE2 (1 μg/L) or vehicle control (VC, ERM containing 0.01% acetone), respectively, in 96 well-microplates (1 egg/well) for a total of 42 days from embryonic, larval to juvenile stages. Mortality and gross development were observed at least once daily. At 6–7 days post-fertilization (dpf), the sex of each embryo was determined under a fluorescence microscope according to genotypic biomarkers [27]. The volume of dosing solutions was increased properly with exposure time and the dosing solution was 80–100% renewed every other day. During chemical exposure, each treatment was in four replicate per concentration (each containing 20–25 fish). The concentrations of the test substance in dosing solutions were determined at appropriate intervals, described as follows. At the end of chemical exposure, all treated fish were transferred to a flow-through glass system with recirculation of clean dechlorinated tap water (without addition of chemicals) and reared until sexually mature. Adult fish (4 months old) were anesthetized for analyses of secondary sex characteristics, and then killed. The liver was used for measurement of liver enzymatic activity, and the middle-posterior segment of the body was used for gonadal histopathology. The remaining fish (F0) were continuously reared until 5 months old for reproductive tests. The gross development, hatchability and genotypic sex of fertilized eggs (F1) were assessed. Then, F0 fish were killed for analysis of gene expression in liver and gonads. Fish were fed with fresh brine shrimp (Artemia sp.) ad libitum (Ocean Star International, INC., U.S.A) twice daily and Otohime β1 (Nisshin Co., Japan) 1 h before water renewal. All chemicals used above were analytical grade (Sigma Aldrich Inc., MO, USA).

2.3. LC–MS/MS analysis of chemical concentration

Each water sample (50 ml) was loaded on a solid-phase extraction (SPE) cartridge (Oasis HLB-columns, Waters, CT, USA) and eluted with 10% MeOH in diethyl ether for chemical extraction according to the manufacturer's instruction. The eluent was dried, and the residue was reconstituted in 25% MeOH. Chromatographic separation of analytes involved use of an Agilent 1200 module (Agilent, CA, USA) equipped with a ZORBAX Eclipse XDB-C18 column (Agilent, 150 mm × 4.6 mm, 5 μm). Mass spectrometry measurements involved use of the Sciex API 4000 instrument (ABI) equipped with an electrospray ionization interface. Analyses were performed in positive mode for LET and negative mode for EE2. Detailed conditions of LC–MS/MS analysis for target compounds are provided

Please cite this article in press as: P.-H. Liao, et al., Persistent endocrine disruption effects in medaka fish with early life-stage exposure to a triazole-containing aromatase inhibitor (letrozole), J. Hazard. Mater. (2014), http://dx.doi.org/10.1016/j.jhazmat.2014.02.013