Aquatic Toxicology 198 (2018) 73–81

Contents lists available at ScienceDirect

Aquatic Toxicology

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

Azoles additively inhibit cytochrome P450 1 (EROD) and 19 (aromatase) in rainbow trout (*Oncorhynchus mykiss*)

Kristina Beijer*, Maria Jönsson, Siraz Shaik, Daphné Behrens, Björn Brunström, Ingvar Brandt*

Environmental Toxicology, Department of Organismal Biology, Uppsala University, Norbyvägen 18A, SE-75236 Uppsala, Sweden

ARTICLE INFO

Keywords:
Cytochrome P450 (CYP) enzyme
Aromatase (CYP19)
Ethoxyresorufin-*O*-deethylase (EROD)
Azole
Inhibition
Fish
Additivity

ABSTRACT

Antifungal azoles are widely used in medicine, agriculture, and material protection and several antifungal azoles have been found in environmental samples. Although these compounds were designed to inhibit fungal enzymes such as lanosterol-14-demethylase (cytochrome P450 (CYP) 51), it is well established that the inhibitory actions of azoles are not specific for fungal CYP isozymes.

We refined a gill filament assay to determine the inhibition of CYP1, measured as reduced 7-ethoxyresorufin-*O*-deethylase (EROD) activity, in rainbow trout (*Oncorhynchus mykiss*) gill tissue *ex vivo*. The advantage of this method is that both induction and inhibition of EROD are performed *ex vivo*. Among thirteen azoles studied, the five that caused the strongest inhibition of gill EROD activity at a concentration of 5 μM were selected for concentration–response assessment. These compounds (bifonazole, clotrimazole, imazalil, miconazole, and prochloraz) showed IC_{50} values ranging from 0.1 to 1.5 μM. CYP19 (aromatase) inhibition was measured using microsomes from rainbow trout brains. Concentration-response curves for CYP19 inhibition were determined for letrozole, bifonazole, clotrimazole, imazalil, miconazole and prochloraz, which gave IC_{50} values ranging from 0.02 to 3.3 μM. It was further found that mixtures of the five most potent azoles reduced both CYP1 and 19 catalytic activity in an additive fashion (IC_{50} = 0.7 μM and 0.6 μM, in the respective assay). Bifonazole (IC_{50} = 0.1 μM) is not previously known to inhibit CYP1 activity.

The additive inhibition of CYP1 and CYP19 catalytic activity is an important finding of the present study. We conclude that this additive action of azoles could mediate adverse impacts on CYP regulated physiological functions in environmentally exposed fish.

1. Introduction

Azole compounds (imidazoles, benzimidazoles, triazoles and benzotriazoles) have broad applications and are for instance extensively used in human and veterinary medicine for treatment of superficial and systemic fungal infections. They are also widely used in agriculture for control of fungi on grain, vegetables and fruit and as biocidal products for material protection such as wood preservation and treatment of paints and concrete. Benzotriazoles are used as anti-icing fluids, corrosion inhibitors and drug precursors.

Fungicidal azoles inhibit fungal cytochrome P450 (CYP) enzymes, e.g. lanosterol-14-demethylase (CYP51), resulting in decreased ergosterol synthesis and loss of fungal cell membrane integrity (Vanden Bossche, 1985). Certain azoles are used as pharmaceuticals for treatment of estrogen-dependent tumors (such as breast cancer) because they inhibit aromatase (CYP19), an enzyme catalyzing formation of estrogens from androgens.

Although azole antifungal agents inhibit CYPs in fungi, it is well established that they may also inhibit various CYP enzymes in vertebrate and invertebrate species. Several CYPs in animals are inhibited by azoles *in vitro*, e.g. CYP11, 17, 19, 21 and 51 (Fernandes et al., 2007; Hinfray et al., 2006; Lamb et al., 1999; Ohlsson et al., 2009; Roelofs et al., 2013; Trösken et al., 2006). Also teleostean CYPs, such as CYP1A and 3A, are inhibited by azole fungicides (Beijer et al., 2010; Burkina et al., 2013; Hasselberg et al., 2005; Hasselberg et al., 2008; Hegelund et al., 2004; Lennquist et al., 2008; Levine et al., 1997; Wassmur et al., 2013).

Limited data are available on the occurrence of azole fungicides in the aquatic environment. A few studies report that azole fungicides are present in low (ng/L) concentrations in rivers, lakes and wastewaters (Kahle et al., 2008; Lindberg et al., 2010; Peng et al., 2012; Stamatis et al., 2010). However, in an EU-wide monitoring of contaminants in wastewater treatment plant effluents it was found that the median concentration of 1H-benzotriazole was as high as 2.7 μg/L (Loos et al., 2013).

We have previously reported that a number of azoles inhibit *7-*

* Corresponding authors.
E-mail addresses: Kristina.Beijer@medsci.uu.se (K. Beijer), Ingvar.Brandt@ebc.uu.se (I. Brandt).

https://doi.org/10.1016/j.aquatox.2018.02.016
Received 4 July 2017; Received in revised form 15 February 2018; Accepted 18 February 2018
Available online 21 February 2018
0166-445X/

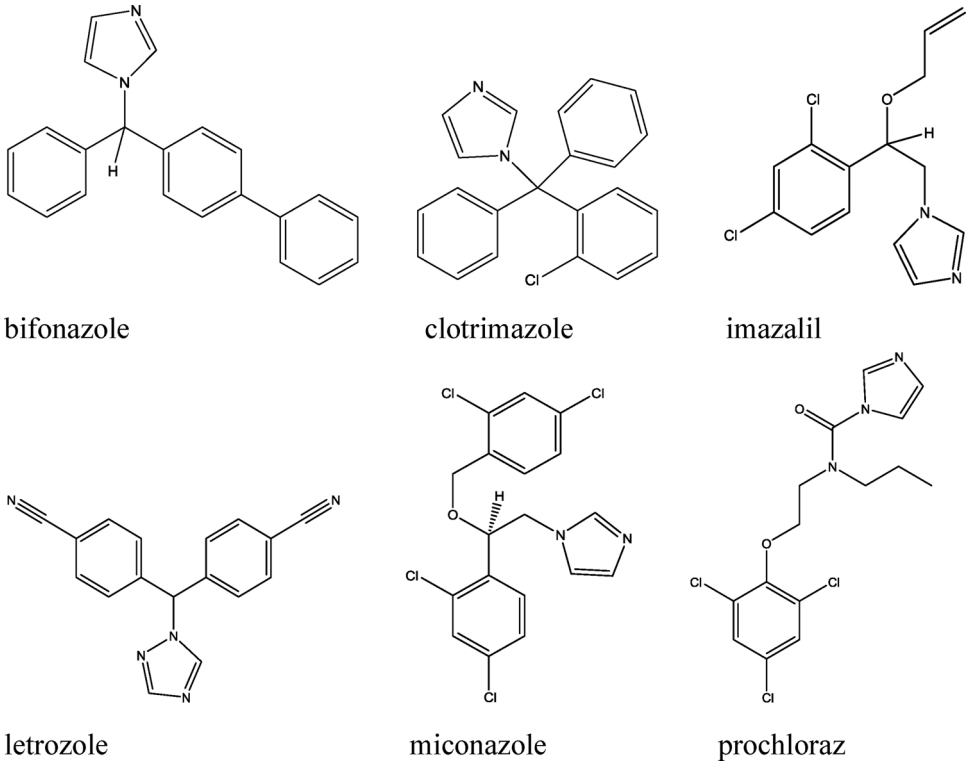

Fig. 1. Structural formulas of the six azoles that were included in mixture experiments. Letrozole was only included in the mixture studied for aromatase inhibition and prochloraz only in the mixture studied for EROD inhibition. From left to right bifonazole, clotrimazole, imazalil (first row), letrozole, miconazole and prochloraz (second row).

ethoxyresorufin-*O*-deethylase (EROD) activity in gill filaments of three-spined stickleback (*Gasterosteus aculeatus*) *ex vivo* (Beijer et al., 2010). Deethylation of 7-ethoxyresorufin is catalyzed by aryl hydrocarbon receptor (AhR)-regulated CYP1 enzymes. Since compounds that activate the AhR induce these enzymes, EROD activity is used as a biomarker for exposure to AhR agonists.

The CYP1 family comprises four subfamilies in fish: CYP1A, CYP1B, CYP1C, and CYP1D (Goldstone et al., 2007; Goldstone et al., 2009). In rainbow trout (*Oncorhynchus mykiss)* six inducible transcripts have been identified; *CYP1A1*, *CYP1A3*, *CYP1B1*, *CYP1C1*, *CYP1C2* and *CYP1C3* (Berndtson and Chen, 1994; Råbergh et al., 2000; Jönsson et al., 2010). CYP1 expression can be highly induced in various tissues in fish including gills (Gao et al., 2011; Jönsson et al., 2006; Ortiz-Delgado et al., 2005; Sarasquete and Segner, 2000; Smolowitz et al., 1991; Smolowitz et al., 1992). CYP1A, the most extensively studied CYP1 form, is involved in the metabolism of a variety of environmental contaminants and in the biotransformation of estradiol and pregnenolone in fish (Petkam et al., 2003; Scornaienchi et al., 2010a). Recent studies show that, besides CYP1A, all CYP1 enzymes can catalyze the deethylation of 7-ethoxyresorufin to some extent (Scornaienchi et al., 2010b; Stegeman et al., 2015). Therefore, CYP1 more precisely defines enzymes that catalyze this reaction.

As mentioned, azoles may inhibit aromatase (CYP19) activity, both in mammals and fish. In fish, aromatase is mainly present in ovary and brain. Many fish species, including rainbow trout, express two *CYP19* genes (*CYP19A* and *CYP19B*), which code for the proteins CYP19A (CYP19A1, aromatase A or P450aromA) and CYP19B (CYP19A2, aromatase B or P450aromB). In rainbow trout, *CYP19B* is predominantly expressed in brain, while *CYP19A* is predominantly expressed in ovary (Tanaka et al., 1992; Valle et al., 2002). Aromatase activity in brain is generally higher in fish than in other vertebrates (Pasmanik and Callard, 1985).

A purpose of the current study was to modify our previously published gill filament EROD inhibition assay (Beijer et al., 2010), to allow both initial EROD induction and subsequent EROD inhibition to be carried out *ex vivo*. This assay and an aromatase assay based on rainbow trout brain microsomes were used to investigate a further objective, which was to study EROD and aromatase inhibition by mixtures of azoles with the hypothesis that azoles act in an additive way.

2. Materials and methods

2.1. Animals

Rainbow trout were purchased from Näs fiskodling AB (By Kyrkby, Sweden) in January and October 2013 and held in the aquarium facility of the Evolutionary Biology Centre, Uppsala University at least two months before experiments started. The fish were kept in flow-through tanks supplied with copper-free tap water (about 12 °C) and were daily fed Inicio 917 or Eficio Alpha 790 pellets (BioMar, Brande, Denmark). The day/night cycle was adjusted automatically to the diurnal variations at latitude 52°N. The fish used in the EROD experiments were 25.3 ± 2.9 cm long (mean ± SD) and weighed 191.4 ± 71.8 g. In the aromatase experiments, fish were 23.4 ± 1.4 cm long and weighed 159.5 ± 30.4 g.

2.2. Chemicals

[1β-3H (N)]androst-4-ene-3,17-dione (purity > 97%, specific activity 24 Ci/mmol) was purchased from PerkinElmer (Boston, MA, USA). 6-Formylindolo[3,2-*b*]carbazole (FICZ; 99.4%, CAS 72922-91-7) was acquired from Syntastic AB, Stockholm, Sweden. Bifonazole (reference standard, CAS 60628-96-8) and letrozole (99.7%, CAS 112809-51-5) were obtained from European Pharmacopeia (Strasbourg, France). 1H-Benzotriazole (≥98%, CAS 95-14-7), 4-methyl-1H-benzotriazole and 5-methyl-1H-benzotriazole (≥90.0%, CAS 29878-31-7), clotrimazole (100%, CAS 23593-75-1), fluconazole (≥98%, CAS 86386-73-4), flusilazole (99.8%, CAS 85509-19-9), imazalil (99.7%, CAS 35554-44-0), ketoconazole (> 98%; CAS 65277-42-1), miconazole nitrate salt (100%, CAS 22832-87-7), prochloraz (98.6%, CAS 67747-