PERGAMON

Journal of Steroid Biochemistry and Molecular Biology 70 (1999) 89–95

The Journal of
Steroid Biochemistry
&
Molecular Biology

www.elsevier.com/locate/jsbmb

Rainbow trout, *Oncorhynchus mykiss*, as a model for aromatase inhibition

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Received 10 July 1998; accepted 22 January 1999

Abstract

The feasibility of utilizing rainbow trout, *Oncorhynchus mykiss*, as an alternative model for studying the inhibition of aromatase (CYP 19) was investigated. The suppression of estrogen-dependent tumors by aromatase inhibitors has been important in the treatment of breast cancer. Estrogens, estrogen precursors and xenoestrogens have been found to promote liver cancer in the trout model. A steroid, 4-hydroxy-4-androstene-3,17-dione (4-OHA), and non-steroids, aminoglutethimide (AG) and Letrozole (CGS 20267), all of which are known aromatase inhibitors in rats and humans, were examined in vitro for activity in trout ovarian microsomes. Aromatase activity was quantified as the release of ^3H_2O from the conversion of [^{3}H]-4-androstene-3,17-dione to 17β-estradiol and estrone. Trout ovarian microsomes exhibited activity between 39–60 fmol mg^{-1} min^{-1} with a calculated V_{max} of 71.1 fmol mg^{-1} min^{-1} when incubated at 25°C with 200 nM 4-androstene-3,17-dione ($K_M = 435$ nM). Significant inhibition by 4-OHA up to 80% was seen at 1.5 μM. At 2000 μM, AG decreased aromatase activity by up to 82%. Letrozole reduced aromatase activity a maximum of 90% in a dose-dependent manner, but the K_i (2.3 μM) was 1000-fold higher than reported in human trials. Indole-3-carbinol and some of its derivatives, two DDE isomers and four flavones (except α-naphthoflavone) at 1000 μM did not significantly inhibit aromatase in vitro. Letrozole and clotrimazole, fed to juvenile rainbow trout at doses up to 1000 ppm for 2 weeks, were not effective in suppressing dehydroepiandrosterone (DHEA) induced increases in vitellogenin and 17β-estradiol levels. These results document that trout aromatase is sensitive to inhibition in vitro by known inhibitors of the mammalian enzyme. The mechanism(s) for lack of inhibition in vivo is currently unknown and must be further investigated in order to develop a trout model for studying the role of aromatase in carcinogenesis. © 1999 Published by Elsevier Science Ltd. All rights reserved.

1. Introduction

Inhibition of estrogen synthesis has become a major focus in the treatment of estrogen-dependent cancers such as breast cancer. Aromatase, the CYP19 gene product, is the enzyme responsible for the conversion of androgens to estrogens, the final step in the estrogen biosynthetic pathway [1–3]. Development of specific inhibitors of aromatase has proven critical for efficacy and safety. General cytochrome P-450 inhibitors, such as aminoglutethimide, would inhibit other steroid cytochrome P-450 biosynthetic enzymes such as those involved in glucocorticoid synthesis and lead to unwanted drug–drug interactions by inhibition of other cytochrome P-450 subfamilies [4–6]. The discovery of highly potent and specific compounds, such as 4-hydroxy-4-androstene-3,17,dione (4-OHA) and Letrozole (CGS 20267), has centered interest on developing aromatase inhibitors that could be used as treatment for breast carcinomas [7–10].

It is possible that some environmental antiestrogens may function, at least in part, through aromatase inhibition. Xenoestrogens have been postulated to play a role in reproductive dysfunction, and in diseases such as cancer, both in wildlife and in humans [11–13]. Lower vertebrate models may prove to be practical alternatives to mammalian models, provided similar

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PII: S0960-0760(99)00090-4

mechanisms of metabolism are characterized. Studies involving reptiles have demonstrated the role aromatase plays in sex determination [14–19]. The presence and effect of naturally occurring and synthetic aromatase inhibitors are not well studied. Natural products and xenobiotics, such as some flavonoids and imidazole fungicides, have been found to inhibit aromatase in rainbow trout ovarian microsomes [20,21]. Rainbow trout have become an established model for carcinogenesis [22,23] and estrogenic pathways for hepatocarcinogenesis have been documented [24]. A crucial advantage is that in vivo studies involving rainbow trout allow for larger sample sizes, providing stronger statistical power at a lower cost than mammalian models.

Our goal was to characterize trout ovarian microsomal aromatase and its sensitivity to inhibitors in vitro and in vivo in order to identify the role of aromatase in estrogen-dependent promotion of hepatocarcinogenesis by compounds such as dehydroepiandrosterone (DHEA) [25–27]. We determined the type of inhibition and K_i values of known inhibitors of human aromatase in trout ovarian microsomes. Several dietary and environmental chemicals were assayed as potential aromatase inhibitors. We also investigated the ability of two compounds, Letrozole, 4-4′-(1-*H*-1,2,4-triazol-1-yl-methylene) bis-benzonitrile, (CGS 20267) and clotrimazole (1-[*o*-chloro-α,α-diphenyl-benzyl] imidazole), an imidazole fungicide, to inhibit aromatase activity in vivo by blocking DHEA induced vitellogenesis.

2. Materials and methods

Letrozole (CGS 20267) was obtained as a gift from Ciba Geigy, Switzerland. [1,2,6,7-^{3}H]-4-Androstene-3,17-dione, specific activity, 93 Ci/mmol, was acquired from Amersham (Buckinghamshire, UK). All other chemicals were purchased from Sigma Biochemicals (St. Louis, MO). Materials for aromatase assay and TLC were purchased from Fisher Scientific (Santa Clara, CA). Enzyme immunoassay (EIA) kits for 17β-estradiol (E_2) and testosterone (T) were developed by Cayman Chemical (Ann Arbor, MI).

Ovaries were removed from mature female rainbow trout, Mt. Shasta strain, euthanized with an overdose of tricane methanesulfonate (MS 222) as approved by the Oregon State University Institutional Animal Care and Use Committee and snap frozen in liquid nitrogen. Microsomes were prepared using a modified method of Guengerich [28]. Tissue was homogenized in ice cold phosphate buffer (0.1 M potassium phosphate, 0.15 M KCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) with a Polytron homogenizer (Brinkman instruments, Westbury, NY). The homogenate was spun at $600 \times g$ for 10 min and the lipid layer was removed. The remaining supernatant was spun for 25 min at $10000 \times g$. The microsomal fraction was obtained by spinning the supernatant at $100000 \times g$ for 95 min. The pellet was washed in 0.1 M potassium pyrrophosphate, pH 7.4, containing 1 mM EDTA, 0.1 mM butylated hydroxytoluene (BHT) and 0.1 mM PMSF. The pellet was resuspended in phosphate buffer containing 30% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF and stored at −80°C. Lyophilized monkey placental microsomes, obtained as a gift from John Resko (Dept. Physiol. and Pharmacol., Oregon Health Sciences University, Portland), were also resuspended in phosphate resuspension buffer.

Aromatase activity was determined using a variation of the method measuring the release of tritiated water from the conversion of [1,2,6,7-^{3}H]-4-androstene-3,17-dione to E_2 [29]. Each incubation mixture consisted of 1 mg protein from mature rainbow trout ovarian microsomes determined by the method of Lowry et al. [30], the desired inhibitor concentration, 1 μCi tritiated 4-androstene-3,17-dione, 200 nM 4-androstene-3,17-dione (androstenedione) and 2 mM NADPH. The reaction mixture was brought to a final volume of 300 μl with phosphate buffer (0.1 M Tris–acetate, 0.1 M KCl, 1.0 mM EDTA, 0.1 mM BHT, pH 7.4) and incubated at 25°C for 1 h while mixing at 100 rpm on an orbital shaker. The conditions were identical for the monkey placental microsomes except that the incubation was carried out at 37°C. The reaction was stopped with 1.7 ml H_20 and 4.0 ml methylene chloride. After vortexing briefly and centrifuging for 10 min at $2000 \times$ g, the aqueous layer was removed and extracted again with methylene chloride. The aqueous layer was stripped of remaining organics with 1% dextran coated charcoal. The mixture was spun at $10000 \times g$ for 10 min and the aqueous layer measured for $^3H_20$ released from the 1β position of androstenedione on a Beckman LS 6500 scintillation counter. The activity was expressed in fmol mg^{-1} min^{-1} based on negative controls containing no NADPH. One way ANOVA and *F*-tests were performed to determine statistical significance of aromatase inhibition compared to positive controls containing vehicle alone.

Thin layer chromatography was used to verify the conversion of [^{3}H]-androstenedione to [^{3}H]-17β-estradiol using an 85:15 dichloromethane:ether solvent system [31]. The organic fraction from the tritiated water assay was concentrated to dryness under a stream of argon gas and resuspended in 100 μl methylene chloride. Using a microsyringe, 10 μl was spotted onto KSF silica gel plates, 60 A, 5 × 10 cm, 250 μm thick, and substrates and products visualized by fluorescence detection under short wave UV light. The E_2 and androstenedione bands were cut out of the plate and

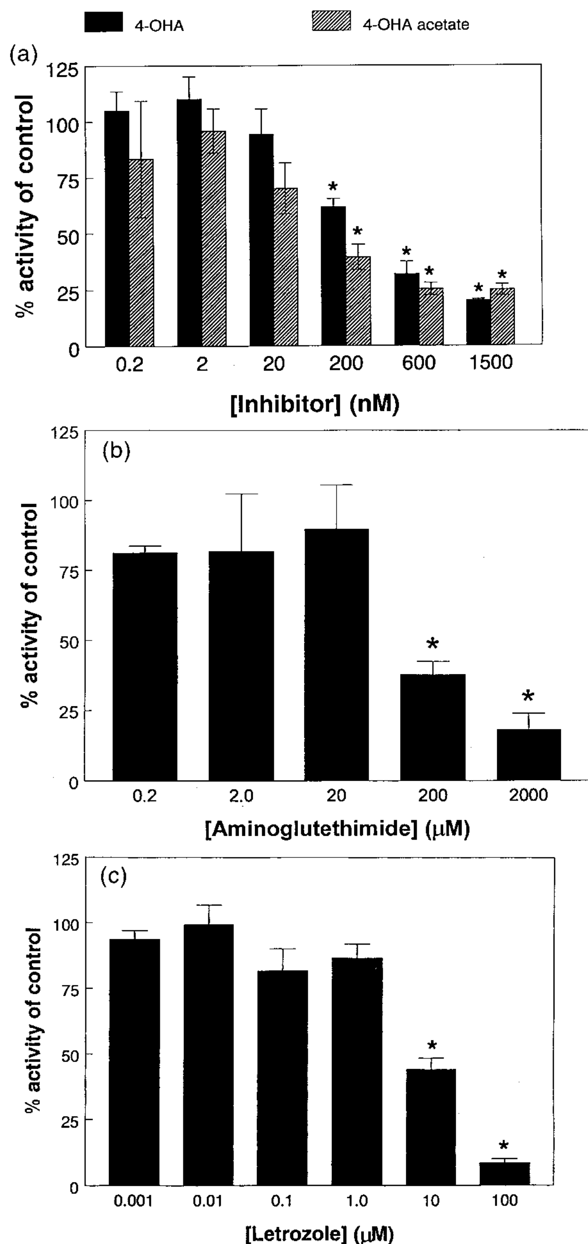

Fig. 1. In vitro inhibition of trout ovarian microsomal aromatase by 4-OHA and 4-OHA acetate (A), aminoglutethimide (B) and Letrozole (C), expressed as percent activity of positive controls. Bars represent ±S.E. ($n = 4$/group). * denotes $p < 0.01$ (ANOVA F-test).

dpm measured to quantify the percent conversion of androstenedione to E_2.

Juvenile rainbow trout, *Oncorhynchus mykiss*, 6 per treatment group, of 50–100 g were randomly allocated into and maintained in 375-l flow through tanks at 14°C with a 12-h light:dark cycle. Control fish were fed a maintenance ration (2.8% wet wt.) of Oregon Test Diet (OTD), a casein based semipurified diet [32]. Test fish received OTD with vehicle containing either 100 ppm DHEA, a high dose of the test compound (1000 ppm Letrozole or clotrimazole) or a combination

Table 1
Comparison of percent inhibition of trout ovarian microsomal aromatase activity by 4-OHA measured by thin-layer chromatography (TLC) and tritiated water (^3H_2O) assay. Values are expressed as percent dpm compared to controls. For TLC analyses, bands corresponding to standards on the plate visualized by UV light were cut out, sonicated in methylene chloride, put in Ultima Gold scintillation cocktail and dpm quantified by scintillation counting (*significant decreases were observed at these concentrations of 4-OHA compared to control ($p < 0.01$, t-test))

Thin-layer chromatography			^3H_2O Assay	
4-OHA (nM)	E_2 (% control)	SE (+/−)	Activity (% control)	SE(+/−)
0	100.00	9.45	100.00	13.04
200	59.49*	3.83	61.90*	3.55
600	25.03*	0.48	31.84*	5.67

of 100 ppm DHEA and test compound for 2 weeks. The vehicles for Letrozole and clotrimazole were dichloromethane and dimethylsulfoxide (DMSO), respectively, which were added to control diets and accounted for less than 0.1% of the diet. Blood samples were drawn from the caudal artery into 3 ml Vacutainer tubes containing 45 USP units of sodium heparin. The protease inhibitors, aprotinin (50 Kallikrein Inhibitory Units (KIU)/ml blood) and EDTA (1 mM) were added to each sample to reduce vitellogenin degradation. Blood was stored on ice until plasma was obtained by centrifugation at 2000 × g for 10 min at 4°C. Plasma was stored at −80°C until later analyses.

Blood plasma vitellogenin, a glycophosphoprotein normally present only in mature females, has been used as a biomarker of estrogenic activity in fish, amphibians and reptiles [33–35]. Vitellogenin concentrations were determined by a modification of a previously described ELISA method [35]. Steroid levels were determined using an EIA method for E_2 and T. Colormetric readings for both immunoassays were performed on a microtiter plate reader (Biotek EL 340, Winooski, VT) and analyzed with plate reader software (Deltasoft 3, Princeton, NJ).

3. Results

Trout ovarian microsomes exhibited aromatase activity ranging between 39 and 60 fmol mg^{-1} min^{-1}, comparable to the 70–80 fmol mg^{-1} min^{-1} observed for the monkey placental microsomes. The K_M and V_{max} for trout ovarian aromatase was calculated to be 435 nM and 71.1 fmol mg^{-1} min^{-1}, respectively. Kinetic analysis revealed that the steroid analogs, 4-hydroxy-4-androstene-3,17-dione (4-OHA) and 4-acetoxy-4-androstene-3,17-dione (4-OHA acetate), displayed mixed inhibition of aromatase with estimated

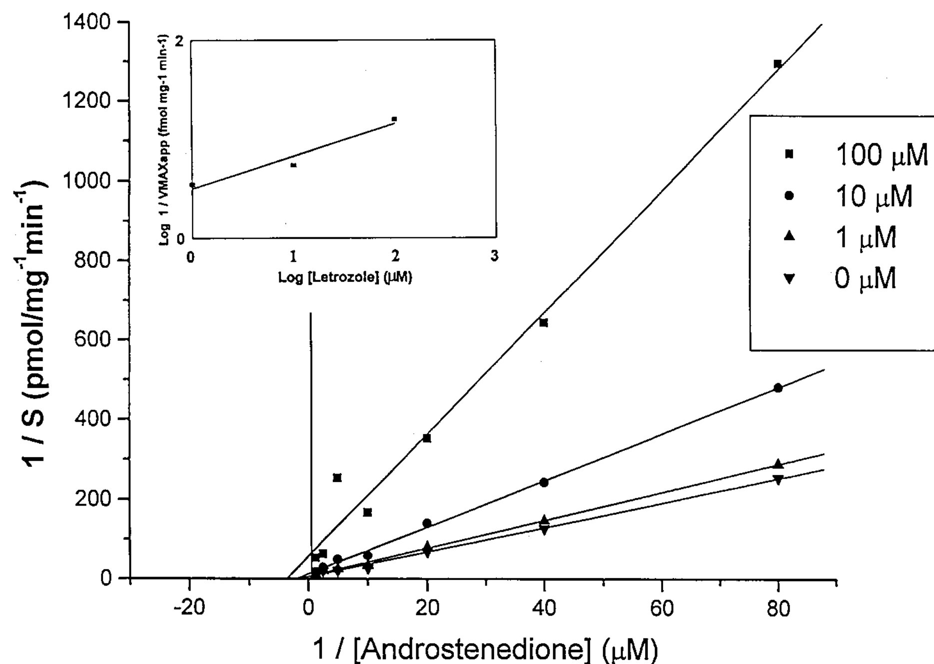

Fig. 2. Lineweaver–Burke plots of Letrozole indicate non-competitive inhibition with androstenedione at concentrations of 12.5–800 nM. The K_i was calculated to be 2.3 μM from the inhibition curve (inset).

K_i values of about 0.2 μM. Maximum inhibition was seen at 1.5 μM at which point aromatase activity was decreased by 80% ($p < 0.01$) (Fig. 1A). Inhibition of trout aromatase by 4-OHA determined by the ^3H_2O assay was similar to values obtained by thin layer chromatography of the organic fraction (Table 1), which measured 17β-estradiol production during the incubation. With an estimated K_i of 300 μM, aminoglutethimide had a potency that was 1000-fold less than 4-OHA, although at 2000 μM, the efficacy for inhibition was similar (82%, $p < 0.01$ (Fig. 1B)). Letrozole significantly inhibited aromatase activity at doses in the micromolar range ($K_i = 2.28$ μM, $p < 0.008$), with a maximal reduction of 90% at 100 μM (Fig. 1C). Interestingly, Letrozole displayed non-competitive inhibition of the rainbow trout ovarian aromatase enzyme (Fig. 2) with a potency that was about 1000-fold less than has been reported for the mammalian enzyme [36,37]. Clotrimazole was found to significantly inhibit ovarian microsomal aromatase activity by up to 92% at concentrations above 10 μM (Fig. 4).

Several dietary and environmental compounds known to have estrogenic and antiestrogenic activities, were screened for aromatase disrupting properties. Indole-3-carbinol, its acid condensation reaction mix-

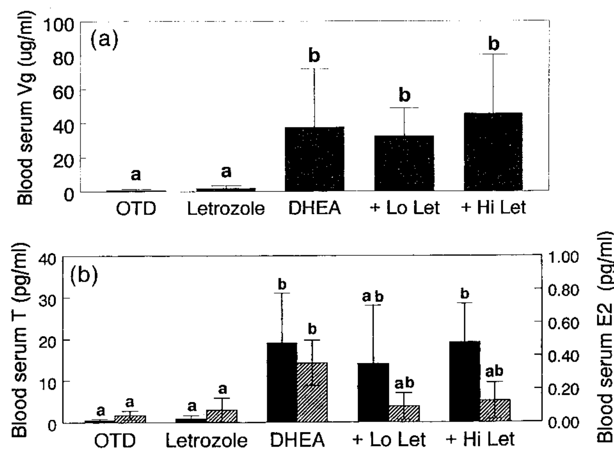

Fig. 3. Blood serum analyses of juvenile rainbow trout fed DHEA and/or a low dose (100 mg/kg/day) or a high dose (1000 mg/kg/day) of Letrozole (A) Blood serum vitellogenin levels: different letters represent significant differences between comparison groups ($p < 0.05$). (B) Blood serum T (solid bars) and E_2 (striped bars) concentrations: different letters represent significant differences between comparison groups ($p < 0.05$). Bars represent ±S.E. ($n = 6$/group).

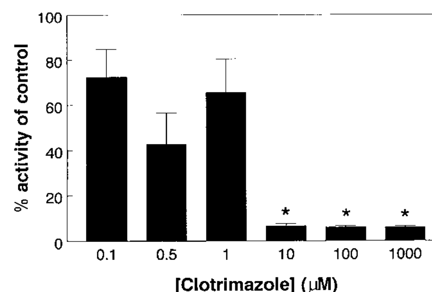

Fig. 4. Dose response of clotrimazole in vitro on aromatase activity of trout ovarian microsomes. Bars represent ±S.E. ($n = 4$/group). * denotes $p < 0.0005$ (ANOVA F-test).

Table 2
Percent inhibition of trout ovarian microsomal aromatase activity by flavones, indoles and DDEs (*α-naphthoflavone significantly inhibited aromatase at 1000 μM ($p < 0.05$, ANOVA F-test). #Induction of aromatase was observed with DDEs, but were not significant ($p > 0.05$, ANOVA F-test))[a]

Chemical	Concs. (μM)	Max inhibition (%control)
Flavones		
Kaempferol	10, 100	91.3 ± 12.8
Apigenin	10, 1000	89.5 ± 2.2
Chrysin	10, 1000	87.1 ± 22.0
α-Naphthoflavone	10, 1000	62.9 ± 10.6*
Indoles		
I3C	1, 10, 100, 1000	92.6 ± 2.1
I33′	1, 10, 100, 1000	78.9 ± 3,1
I3C rxn mixture	1, 10, 100, 1000	85.9 ± 8.1
DDEs		
o,p′-DDE	100, 1000	105.0 ± 14.8#
p,p′-DDE	100, 1000	146.3 ± 15.7#

[a] Abbreviations: Indole-3-carbinol (I3C), 3,3′-diindolylmethane (I33′).

ture products [38], and purified dimer, 3,3′-diindolylmethane, had no effect on aromatase activity at concentrations up to 1000 μM (Table 2). Our laboratory had previously shown that 3,3′-diindolylmethane was an effective inhibitor of trout, rat and human drug-metabolizing cytochrome P450s, with K_is in the low micromolar range [39]. Neither *o,p′*- nor *p,p′*-DDE altered aromatase activity at the highest concentrations. Flavone derivatives have been shown to inhibit aromatase in human preadipocytes and ovarian and placental microsomes [40,41]. α-Naphthoflavone significantly inhibited trout ovarian aromatase at concentrations of 1000 μM, but only to a maximum reduction of 40% compared to controls (Table 2). Other flavones tested, including chrysin, apigenin and kaempferol, did not inhibit aromatase at concentrations up to 1000 μM (Table 2).

An in vivo study was conducted to analyze vitellogenin suppression by Letrozole in DHEA treated juvenile trout. Our laboratory had previously documented induction of vitellogenin in trout by DHEA [42]; an estrogenic mechanism of DHEA may be responsible for its promotion of liver cancer in the trout model [25–27,42]. In this study, we observed a 60-fold induction of vitellogenin after 2 weeks of feeding with 100 ppm DHEA in both males and females ($p < 0.0001$). There was no observed sex difference in either the control or the DHEA treated group. Letrozole did not significantly decrease DHEA-induced vitellogenin production at doses of 100 and 1000 ppm ($p = 0.14$) (Fig. 3A). E_2 and T production was increased by 8- and 34-fold ($p < 0.0002$ and $p < 0.0001$), respectively, by DHEA as determined by EIA analysis (Fig. 3B). The levels of E_2 in DHEA treated trout cotreated with Letrozole decreased, suggesting an inhibition of aromatization. This decrease, however, was not statistically significant ($p = 0.07$).

Clotrimazole was tested for inhibiting properties in vivo. It was not an effective inhibitor of DHEA induced E_2 plasma levels at doses up to 1000 ppm, consequently, vitellogenin production was not inhibited significantly either (data not shown).

4. Discussion

Aromatase activity observed in mature female rainbow trout ovarian microsomes was comparable to that seen in mammalian estrogenic tissues such as monkey placenta. Trout ovarian microsomes proved to be a useful model for discovering effective in vitro aromatase inhibitors. Several compounds of varying structures were found to be effective and potent aromatase inhibitors in vitro. The most potent inhibitor in vitro was 4-OHA, a steroid analog of androstenedione, which has been demonstrated to be an irreversible inhibitor in primates [45]. Aminoglutethimide, a compound used to treat estrogen-dependent breast cancer, was equally effective at inhibiting aromatase activity in vitro compared to 4-OHA, but required a 200-fold higher dose for this response. Our in vitro data support the concept that the rainbow trout aromatase enzyme itself is similar to other species in activity, but at least in the case of Letrozole, the mechanism and efficacy of inhibition are different.

Two promising orally bioavailable compounds, Letrozole and clotrimazole, were tested further for the possibility of inhibiting aromatase in vivo. Neither Letrozole nor clotrimazole blocked vitellogenin production or E_2 synthesis in vivo, at doses up to 1000 ppm. Based on our in vitro data and previous data reported in mammalian studies, these results were unexpected. This suggests that rainbow trout may exhibit a species difference compared to humans for aromatase inhibition or that there are pharmacokinetic reasons these two compounds are not reaching the target organ at levels sufficient for inhibition. E_2 levels tended to be lower with Letrozole treatment in vivo, suggesting inhibition of aromatizing androgens. The high variability between individual fish, a common observation because they are not as inbred as mammalian biological models, may have accounted for the lack of an observed significant decrease ($p = 0.07$).

There were differences in activity for Letrozole in trout aromatase compared to mammals. Bioavailability is not an issue when incubating with ovarian microsomes, and perhaps there is a difference in metabolism and absorption of these chemicals in vivo compared to mammals. To increase sensitivity, juvenile

trout (< 18 months) were experimental subjects, because at this age they have very low circulating E_2, T and vitellogenin levels; juvenile trout fed the proandrogen DHEA respond with elevated levels of all three parameters. As estrogens are known liver tumor promoters in trout, our goal was to evaluate the role of E_2 synthesis in DHEA carcinogenesis by aromatase inhibition in vivo. The dose of DHEA used was based on tumor study data and was perhaps too high for vitellogenin and steroid inhibition studies. By looking at inhibition of endogenous steroids in mature fish, confounding variables involving DHEA cotreatment would be eliminated, possibly revealing aromatase inhibiting activity. Unlike humans, rainbow trout do not have appreciable circulating levels of this androgen precursor in their blood [43,44]. There is the potential for unique metabolic pathways in rainbow trout to upregulate aromatase, counteracting any inhibitory effect, to enhance elimination, or to render the inhibitor unavailable to target organs by binding to free inhibitor or metabolizing it to an inactive form. Future studies to determine the role of gonadotropins and serum binding proteins will hopefully shed light on this issue.

Although 4-OHA was a potent in vitro aromatase inhibitor, this compound was not tested in vivo because it requires dosing via intramuscular injection and the goal of this study was to find an effective aromatase inhibitor that could be administered orally for long term tumor studies. Due to the low specificity of aminoglutethimide towards inhibiting aromatase, corticoid cotreatment would have been necessary for an in vivo study, thereby introducing confounding variables. This is because in mammalian models it has be documented to suppress adrenal function, specifically 18-hydroxylase, the enzyme that converts corticosterone to aldosterone, with a higher specificity than aromatase [46].

Optimizing an established assay used to quantitate aromatase activity in mammalian microsomes, aromatase activity in rainbow trout ovaries was determined in vitro to be similar to mammals. Known mammalian inhibitors were largely successful in blocking in vitro E_2 synthesis in rainbow trout ovarian microsomes, although in the case of Letrozole, which was 1000 times less potent in trout compared to mammals, the activities were different. This demonstrates that the rainbow trout model is suitable for evaluating potential aromatase inhibitors in vitro. Caution must be used when extrapolating from in vitro results to whole animal effects as shown by the ineffectiveness of Letrozole and clotrimazole to inhibit estrogen synthesis in vivo. The rainbow trout is still a very good candidate for modeling aromatase inhibition with respect to screening environmental chemicals. The mechanisms of endocrine modulating compounds that alter estrogen synthesis in aquatic and terrestrial animals can also be investigated using the rainbow trout. It seems likely that some estrogenic and antiestrogenic compounds act directly on the aromatase enzyme either by inhibition or induction, and this method is a quick means for the screening of such compounds. It is advantageous to discover species differences because it will allow for a better understanding of mechanisms and evolution of the aromatase enzyme. These limited in vivo studies do not support the use of rainbow trout as a model for human aromatase inhibition without further development, but they can be used as an environmental model and potentially for endocrine-dependent cancers in the future.

Acknowledgements

The authors thank Greg Gonnerman, John Kelly, Eric Johnson and Laura Martini at the Food Toxicology National Laboratory for their feeding and care of the trout. This work was supported by NIH grants ES-07060, ES-03850 and ES 04766.

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