

Highlighted article

Comparison of fathead minnow ovary explant and H295R cell-based steroidogenesis assays for identifying endocrine-active chemicals

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Abstract

An in vitro steroidogenesis assay using H295R human adenocarcinoma cells has been suggested as a possible alternative to gonad explant assays for use as a Tier I screening assay to detect endocrine active chemicals capable of modulating steroid hormone synthesis. This study is one of the first to investigate the utility of the H295R assay for predicting effects and/or understanding mechanisms of action across species and tissues. Six chemicals, including one selective aromatase inhibitor (fadrozole), four fungicides (fenarimol, ketoconazole, prochloraz, and vinclozolin), and one herbicide (prometon), were tested in both the H295R steroidogenesis assay, and an in vitro steroidogenesis assay using fathead minnow ovary explants. All six chemicals caused significant alterations in 17 β -estradiol (E2) and/or testosterone (T) production in vitro. Effects of ketoconazole, prochloraz, and prometon were similar in both assays. However, there were differences in the profile of responses for T for fadrozole and fenarimol, and for T and E2 for vinclozolin. In terms of sensitivity, steroid production in the H295R assay was most sensitive for detecting the effects of fadrozole, fenarimol, and prochloraz, but was less sensitive than the fathead minnow ovary explant assay to the effects of ketoconazole and vinclozolin. The H295R assay was consistently less variable (among replicates) than the fathead minnow ovary explant assay. However, the ovary explant assay was more predictive of in vivo effects of the six chemicals on fathead minnows than the H295R system. Further characterization of autoregulatory capacities, interaction of steroid-hormone receptor pathways with steroidogenesis, and metabolic capabilities of each system are needed for either system to provide clear and informative insights regarding a chemical's mechanism of action. Overall, however, results of this study suggest that both the H295R and fathead minnow ovary explant assays have utility for identifying endocrine-active chemicals in screening-type applications.

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1. Introduction

Steroid hormones play critical roles in regulating development, growth, and reproduction in vertebrates (Norris, 1997). Steroids are synthesized from cholesterol

through a series of biochemical reactions mediated primarily by nine different cytochrome P450 (CYP) enzymes and several hydroxysteroid dehydrogenases (HSDs; Fig. 1; Miller, 1988, 2005; Agarwal and Auchus, 2005). Activities of these steroidogenic enzymes can be influenced by a variety of xenobiotic chemicals. For example, the activity of cytochrome P450 aromatase (CYP19), the enzyme involved in conversion of C19

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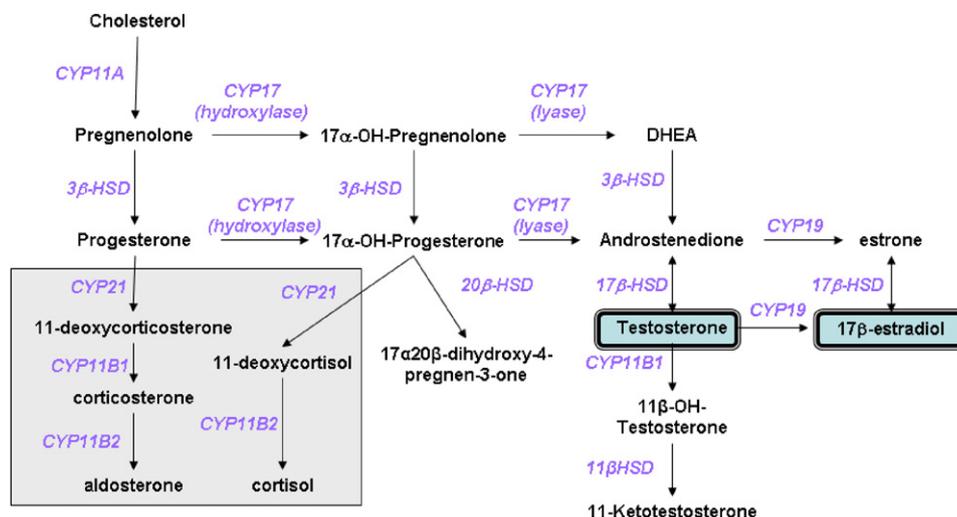


Fig. 1. Major steps involved in steroid hormone biosynthesis. Abbreviations for enzyme names are shown in *italics*. CYP = cytochrome P450; HSD = hydroxysteroid dehydrogenase. Normal background indicates major pathways/products of fish gonad tissue. Gray background indicates predominantly adrenal pathways/products. Hormones measured in this study are circled.

androgens to C18 estrogens, has been shown to be inhibited by a number of fungicides, the acaricide dicofol, certain polychlorinated dibenzo-*p*-dioxins and polychlorinated biphenyls, some organotins, and benzo[*a*]pyrene, and can be induced, at least in vitro, by the fungicide vinclozolin and certain triazine herbicides (Drenth et al., 1998; Letcher et al., 1999; Vinggaard et al., 2000; Sanderson et al., 2001, 2002; Cooke, 2002; Heneweer et al., 2004). Imidazole fungicidal drugs such as ketoconazole, econazole, and miconazole, inhibit steroidogenic CYPs such as cytochrome P450 c17 α hydroxylase, 17,20-lyase (CYP17) (Kan et al., 1985; Van Cauteren et al., 1989; Walsh et al., 2000). Polycyclic aromatic hydrocarbons have also been reported to inhibit ovarian steroidogenesis, likely through inhibition of CYP17, CYP19, and 17 β -HSD (Monteiro et al., 2000). Finally, a variety of plant-derived flavonoids inhibit 3 β -HSD (Wong and Keung, 1999; Ohno et al., 2002, 2004). Thus, there is a wide range of chemicals that exhibit some capacity to modulate normal endocrine function by altering the activities of steroidogenic enzymes. Given the importance of steroid hormones in regulating biological function, under some circumstances such modulation may result in disruptions that could adversely affect development, growth, and/or reproduction.

In 1995 and 1996 the US Congress passed legislation that charged the Environmental Protection Agency (EPA) with developing and implementing a screening program designed to assess whether pesticides and other chemicals to which a widespread population could be exposed could have estrogenic or other endocrine-mediated effects (Bill number S. 1316 Safe Drinking Water Act Amendments of 1995, Bill number PL 104-170 Food Quality Protection Act of 1996). In 1998 an EPA advisory committee submitted their recommendations for a multi-tiered endocrine disruptor-screening program. A rodent minced testis assay using in vitro testosterone (T) production as a primary

endpoint was proposed as Tier I screen to detect chemicals with potential to disrupt steroidogenesis (EDSTAC, 1998; Powlin et al., 1998). However, questions have been raised about high rates of false positive or negative responses observed in mammalian gonad explant assays (Powlin et al., 1998). In addition, use of minced testis or ovary explant assays ignores a suite of enzymes and reactions that are critical for adrenocortical steroidogenesis and the production of glucocorticoids and mineralocorticoids, which are arguably, as important for organism health as the reproductive steroids (Harvey and Everett, 2003).

An in vitro steroidogenesis assay using H295R human adrenocarcinoma cells has been suggested as a possible alternative to the minced testis assay for Tier I screening (Harvey and Everett, 2003; Hilscherova et al., 2004; Zhang et al., 2005; Hecker et al., 2006). H295R cells have physiological characteristics of zonally undifferentiated fetal adrenal cells (Gazdar et al., 1990; Rainey et al., 1993; Staels et al., 1993) and express the enzymes needed to produce the entire complement of steroids synthesized by the adult adrenal cortex, including the glucocorticoids, mineralocorticoids, and reproductive steroids (Gazdar et al., 1990; Staels et al., 1993; Harvey and Everett, 2003). The cells have been used to evaluate effects of chemicals on hormone production, steroidogenic enzyme activities, and steroidogenic gene expression (Sanderson et al., 2000; Hilscherova et al., 2004; Zhang et al., 2005; Hecker et al., 2006). Furthermore, well-defined methods for assessing possible cytotoxicity of test chemicals to the H295R cells are available. Finally, use of the H295R-based assay would obviate the use of animals for such screening. Thus, the H295R assay appears to hold promise as a substitute for gonad explant assays in screening for endocrine-active chemicals (EACs), as well as an alternative to explant assays for research applications.

However, if H295R-based bioassays are to be used for these purposes, it is important to investigate and define their predictive capabilities among species and tissues. Evaluation of the H295R assay as a potential Tier I screening assay for the endocrine disruptor screening and testing program and an assessment of its predictive capabilities relative to mammalian models and/or differentiated adrenal tissue, will be covered elsewhere. The aim of this study was to compare a fathead minnow ovary explant steroidogenesis assay, adapted from the methods of McMaster et al. (1995), to the H295R cell-based steroidogenesis assay described by Hecker et al. (2006), using six chemicals (fadrozole, fenarimol, ketoconazole, prochloraz, prometon, and vinclozolin) known or suspected to affect steroidogenesis *in vivo*. Results of the two *in vitro* assays were also compared to results of a short-term fathead minnow reproduction test that has been developed as an *in vivo* Tier I screening assay for EACs (US Environmental Protection Agency (US EPA), 2002). Consequently, in addition to assessing the comparative sensitivity and variability of the two *in vitro* assays, comparison of the *in vitro* responses to the effects observed in fathead minnows exposed to each of the test chemicals *in vivo* for 21 d was used to evaluate whether either of the *in vitro* assays has clear advantages for predicting *in vivo* outcomes in fathead minnows (and potentially other fish) or for defining functional linkages between mechanism of action and apical responses.

2. Materials and methods

2.1. Chemicals

Fenarimol (99.8% purity), ketoconazole (99% purity), and prochloraz (99.5% purity), were obtained from Sigma-Aldrich (St. Louis, MO, USA). Prometon (technical grade, 98.7% purity, Lot 0310070) was obtained from Platte Chemical (Greenville, MS, USA). Fadrozole was provided by Novartis, Inc. (Summit, NJ, USA). Vinclozolin (99% purity) was purchased from Chem Service (West Chester, PA, USA). Primary H295R assay data for fadrozole, ketoconazole, prochloraz, and vinclozolin have been reported elsewhere (Hecker et al., 2006), but were re-analyzed and expressed in terms of relative change (%) to facilitate comparison to the fathead minnow ovary explant assay results.

2.2. H295R assay

2.2.1. Cell culture

The H295R human adrenocortical carcinoma cells were obtained from the American Type Culture Collection (ATCC CRL-2128; ATCC, Manassas, VA, USA) and were grown in 100 mm² Petri dishes with 12.5 mL of supplemented media at 37 °C with a 5% CO₂ atmosphere as described previously (Hilscherova et al., 2004). Briefly, the cells were grown in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (DMEM/F12; Sigma [D-2906], St. Louis, MO, USA) supplemented with 1.2 g/L Na₂CO₃, 5 mL/L of ITS+ Premix (BD Bioscience [354352], San Jose, CA, USA) and 12.5 mL/L of BD Nu-Serum (BD Bioscience [355100]). The same batch of Nu-Serum was used for all experiments except prochloraz and there was no evidence to suggest that possible variation in the low background concentrations of steroids among batches of Nu-Serum significantly influenced the assay results.

2.2.2. Experimental design

All experiments were conducted in 24-well cell culture plates (COSTAR, Bucks, UK). One mL of cell suspension, at a concentration of $\approx 200,000$ cells/mL, was added to each well and the cells were allowed to attach for 24 h. After the attachment period the medium was changed and the experiment was initiated. Cells were exposed for 48 h in the same 24-well plates. Dimethyl sulfoxide (DMSO) was used as a carrier at a final concentration of 0.1% v/v. Test plates included six chemical concentrations, an appropriate solvent control (SC) and a blank control (CTR), in triplicate. At the end of each experiment, medium was transferred to an Eppendorf tube and stored at -80 °C. Cell viability was evaluated in CTR, SC, and at least the three greatest exposure concentrations of each chemical using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) bioassay (Mosman, 1983).

2.2.3. Hormone measurements

Frozen samples were thawed on ice and extracted twice with diethyl ether (5 mL) in glass tubes. Phase separation was achieved by centrifugation at 2000g for 10 min and the ether phases were collected and combined. The ether was evaporated under a stream of nitrogen and the residue was dissolved in ELISA (enzyme-linked immunosorbent assay) buffer and was either immediately measured or frozen at -80 °C for later analysis. Hormones extracted from the media samples were measured by competitive ELISA using manufacturer recommendations (Cayman Chemical Company, Ann Arbor, MI, USA; testosterone (T) [Cat # 582701], 17 β -estradiol (E2) [Cat # 582251]). Extracts of medium were diluted 1:2, 1:5 and 1:10 for E2, and 1:50, 1:100 and 1:150 for T to use in the ELISAs.

2.3. Fathead minnow ovary explant assays

2.3.1. Experimental design

The fathead minnow *in vitro* steroidogenesis assay used in this study was adapted from the methods of McMaster et al. (1995). Assays were conducted in 48-well microplates (Falcon 35-3078, Beckton Dickinson, Franklin Lakes, NJ, USA). Dilutions of fenarimol and prochloraz were prepared in ethanol (100%, molecular biology grade, Sigma) and prometon dilutions were prepared in methanol (99.99% purity, HPLC grade, Fisher Chemical, Fair Lawn, NJ, USA). Fadrozole, ketoconazole, and vinclozolin stock solutions did not require a solvent (concentrations verified analytically), so dilutions were prepared directly in supplemented culture medium (Medium 199 [Sigma M2520] containing phenol red, supplemented with 0.1 mM IBMX [3-isobutyl-1-methylxanthine, Sigma I7018] and 1 μ g/mL 25-hydroxycholesterol [Sigma H1015]). Supplemented medium and test chemicals were added to the wells of the 48-well test plate prior to adding ovary explants. For chemicals in solvent (i.e., fenarimol, prochloraz, prometon), plates were dosed by adding 5.0 μ L of the appropriate concentration of test chemical, solvent alone, or non-supplemented Medium 199 (control) directly to wells containing 495 μ L of supplemented Medium 199. Four chemical concentrations, a SC, and a control were tested on each plate. In the case of chemicals diluted directly in supplemented medium (i.e., fadrozole, ketoconazole, and vinclozolin), plates were dosed by adding 500 μ L of chemically treated supplemented medium (or supplemented medium alone, controls) to appropriate wells. Five chemical concentrations and a control were tested on each plate. Regardless of dosing, at least eight replicate wells were tested per treatment.

After preparing the test plate (s), ovarian tissue from female fathead minnows was added. A total of eight replicate explants were tested per treatment. Since it was generally not possible to obtain enough ovary tissue from a single fish to provide $n = 48$ 10–20 mg pieces of ovary, tissue from two different fish was used for each experiment. Equal numbers of explants from each fish were exposed to each treatment (i.e., $n = 4$ explants of ovary per fish, per test concentration, for a total of $n = 8$ explants exposed per treatment) and steroid production was normalized to controls on a fish-specific basis in order to normalize for variations in steroidogenic status among individual fish.

All fish were 5–6 months old and were obtained from the on-site aquatic culture unit at the US EPA Mid-Continent Ecology Division (operated by Wilson Environmental). Individual fish were anesthetized in MS-222 (tricaine methanesulfonate; 100 mg/L buffered with 200 mg NaHCO₃/L; Finquel, Argent, Redmond, WA, USA). Ovaries were removed and immediately placed in a Petri plate containing ice-cold non-supplemented Medium 199. The pair of ovaries from each fish was cut into 24 pieces (approx. 10–20 mg) in the ice-cold media and then the pieces were randomly assigned to 24 wells on one-half of the 48-well test plate, on ice. Pieces of ovary from a different fish were randomly distributed to wells on the other half of the test plate. Once ovary tissue had been added to all wells, the test plate was removed from the ice and incubated at 25 °C overnight (14.5 h). At the end of the incubation period, medium from each well was transferred to a microcentrifuge tube and frozen at –80 °C. Medium samples were stored frozen at –20 °C until extracted and analyzed. After removal of the medium, the tissue in each well was removed with a forceps and wet weight was measured. Due to the difficulty associated with determining the viability of specific cell types within a whole tissue explant no measurement of viability was made beyond visual observation of the tissue and pH changes in the phenol-red containing medium. No obvious toxicity was evident at the exposure concentrations tested, however, subtle and/or cell-type specific toxicity cannot be ruled out as a potential factor in the ovary explant assay.

2.3.2. Hormone measurements

Testosterone and E2 concentrations in the medium samples were measured by radioimmunoassay using an adaptation of methods described previously for fathead minnow plasma samples (US EPA, 2002). Briefly, medium samples were allowed to thaw at room temperature and then 400 µL of each sample was transferred to a glass test tube. Excess medium was pooled together, and then divided into separate 400 µL aliquots to serve as quality assurance (QA) samples for evaluating intra-assay variability. Experimental and QA samples were extracted twice by liquid:liquid extraction with diethyl ether (Sigma-Aldrich 309958). Aqueous layers were frozen by placing the sample in a –80 °C freezer or on dry ice for ≥ 15 min and the ether layers were then transferred to a new tube. The two ether extracts were pooled together and allowed to evaporate at room temperature overnight. The following day, samples were resuspended in 200 µL buffer (0.01 M phosphate buffered saline, pH 7.4, with 1% bovine serum albumin [Sigma A7888]). Resuspended samples were split into two 100 µL aliquots and one aliquot was analyzed for T and the other for E2. Duplicate T or E2 standard curves consisting of six concentrations ranging from 0.0247 to 6 ng/mL, prepared by 3-fold serial dilution, were tested with each set of 48 samples (plus corresponding QA samples). Duplicate measures of non-specific binding and maximum-binding tubes were made with each set of 48 samples.

2.4. Data analysis

To facilitate comparison among the two assays, and to normalize for variations in basal hormone production among different batches of H295R cells or different fathead minnows, measured hormone concentrations were normalized to the mean SC (for all treatments using a solvent) or control (for aqueous treatments) and expressed as relative change (%). H295R results were normalized to the mean SC value for each assay (i.e., each 24-well plate of cells used to test a given chemical). Fathead minnow results were normalized to the mean control or SC on a per fish basis. For statistical analysis, a Kolmogorov–Smirnov test was used to test data for normality. Bartlett's test was used to test homogeneity of variance. When parametric assumptions were met, analysis of variance (ANOVA) was used to test for differences across all treatments. A non-parametric Kruskal–Wallis test was used in cases where the data or transformed data did not conform to parametric assumptions. A parametric Dunnett's test or non-parametric Dunn's test was used to determine which treatments differed significantly from the SC or control. All statistical analyses were conducted using SAS 9.0 (SAS Institute, Cary, NC, USA), except Dunn's test which was conducted using GraphPad Instat[®] v. 3.01 (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Fadrozole

Fadrozole impacted E2 production in both assays, but its effect on T differed between the two systems. In the fathead minnow ovary explant assay, fadrozole caused a significant, concentration-dependent, reduction in E2 production at concentrations ≥ 9.7 µM (Fig. 2). Testosterone production was more variable among individual explants and was not significantly affected by fadrozole treatment (Fig. 2). In the H295R assay, fadrozole concentrations, as great as 100 µM, did not significantly alter cell viability. Nonetheless, steroid production was affected. In the H295R cell bioassay, fadrozole caused a significant, concentration-dependent, reduction in E2 production at concentrations ≥ 0.1 µM (Fig. 3; Hecker et al., 2006). However, in contrast to the fathead minnow ovary assay in which there was no effect on T production, exposure to 100 µM fadrozole caused a significant decrease in T production by the H295R cells (Fig. 3; Hecker et al., 2006). The mean T production data was suggestive of a concentration-dependent decrease, but, as in the explant assay, T production by the fadrozole-exposed H295R cells was more variable than E2 production. Overall, the H295R assay was more sensitive to the effects of fadrozole than the ovary explant assay.

3.2. Fenarimol

Fenarimol exposure reduced E2 and T production by fathead minnow ovary explants and H295R cells, relative to controls, but the concentration-response and variability profiles were not consistent between both assays. In the fathead minnow ovary explant assay, T production was affected at all concentrations tested (1.12–30.2 µM) and variability among explants was low (Fig. 2; Table 1). Mean E2 production among replicate fathead minnow explants exhibited a trend similar to that observed for T production, but variability in E2 production was greater (Table 1), such that a statistically significant decrease was only detected at the 30.2 µM treatment (Fig. 2). In the H295R assay, at concentrations > 1 µM, T production data were suggestive of a concentration-dependent decrease in T, but the effect was only significant at the greatest concentration tested (100 µM; Fig. 3). This was also the only fenarimol concentration that significantly reduced cell viability. In contrast, E2 concentrations in the medium were significantly less than those for controls at nearly all concentrations tested (≥ 0.01 µM), and the magnitude of the effect was fairly stable (40–60% SC) over a 100,000-fold range of exposure concentrations (Fig. 3). Overall, the H295R cells were more sensitive to fenarimol's effects on E2 production, while the fathead minnow ovary explant assay was more sensitive to its impact on T.

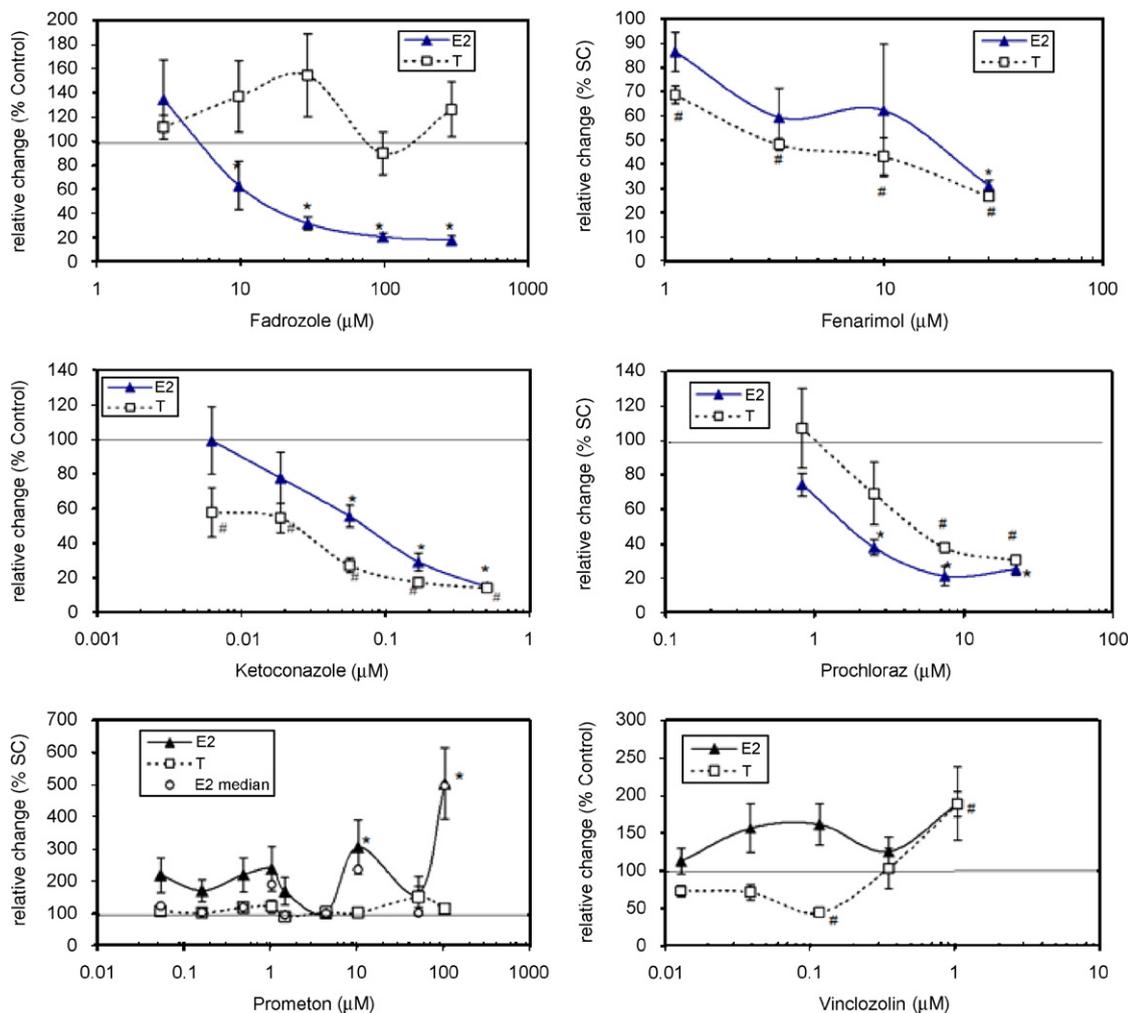


Fig. 2. Concentration–response curves for estradiol (E2) and testosterone (T) production by fathead minnow ovary explants exposed to six test chemicals in vitro. All data expressed as relative change (i.e., percent of the average production by control or solvent control (SC) explants, as appropriate). Error bars = standard error (SE). * indicates E2 production was significantly different from controls ($p < 0.05$). # indicates T production was significantly different from controls ($p < 0.05$). Solid horizontal line = 100%.

3.3. Ketoconazole

Ketoconazole significantly reduced both E2 and T production by fathead minnow ovary explants and H295R cells. In the fathead minnow assay, all concentrations tested (0.006–0.506 μM) significantly reduced T production and the effect became more pronounced at greater concentrations (Fig. 2). Similarly, mean E2 production by the ovary explants showed a steady decline with increasing concentration, and the effect was significant at concentrations $\geq 0.056 \mu\text{M}$ (Fig. 2). In the H295R assay, the effects on T and E2 production were significant at $\geq 1.0 \mu\text{M}$ and $\geq 3.0 \mu\text{M}$, respectively (Fig. 3; Hecker et al., 2006) and none of the ketoconazole concentrations tested were toxic to the H295R cells. Although the concentration–response profiles were similar in both assays, ovary explants were more sensitive to the effects of ketoconazole on E2 and T production than the H295R cells.

3.4. Prochloraz

Prochloraz had similar effects on E2 and T production in both assays. Parallel reductions in T and E2 production were observed for fathead minnow ovary explants exposed to prochloraz (Fig. 2). Prochloraz had a more potent effect on E2 production, causing a 50% reduction at approximately 1.6 μM , and a significant decrease at 2.5 μM (Fig. 2). Based on the concentration–response curve, exposure to approximately 3.5 μM prochloraz caused a 50% reduction in T production, and a statistically significant effect was observed at 7.4 μM (Fig. 3). H295R assay results were similar in that both E2 and T production were reduced in a concentration-dependent manner and the effect on E2 was more pronounced and potent than that on T (Fig. 3; Hecker et al., 2006). Prochloraz was toxic to H295R cells, but only at concentrations $\geq 10 \mu\text{M}$, considerably greater than those that reduced E2 and T production. Concentrations $\geq 0.01 \mu\text{M}$ resulted in

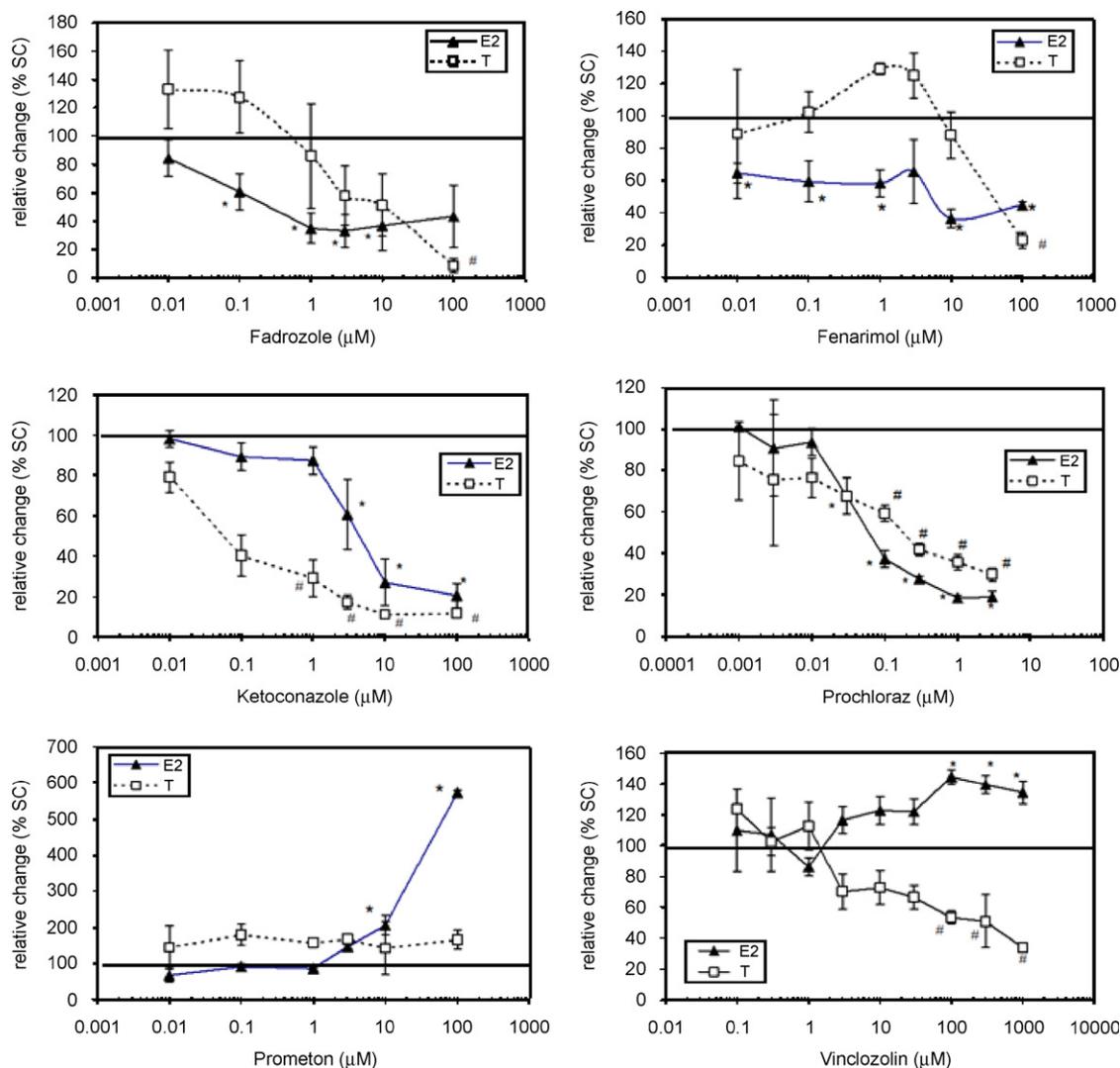


Fig. 3. Concentration–response curves for estradiol (E2) and testosterone (T) production by H295R cells exposed to six test chemicals. All data expressed as relative change (i.e., percent of the average production by cells treated with carrier solvent only [SC, solvent control]). Error bars = standard deviation (SD). * indicates E2 production was significantly different from controls ($p < 0.05$). # indicates T production was significantly different from controls ($p < 0.05$). Solid horizontal line = 100%. Results for fadrozole, ketoconazole, prochloraz, and vinclozolin were reported previously (Hecker et al., 2006) but were re-analyzed and provided here to facilitate comparison to fathead minnow ovary explant results.

significantly less E2 production relative to controls, while those $\geq 0.1 \mu\text{M}$ resulted in significantly less T production (Fig. 3; Hecker et al., 2006). H295R cells were about 100-fold more sensitive to the reduction in E2 and T production than the ovary explants.

3.5. Prometon

Prometon yielded similar concentration–response relationships in both the fathead minnow ovary explant assay and the H295R in vitro steroidogenesis assay. Testosterone production by fathead minnow ovary explants was not affected by exposure to prometon, but E2 production by explants exposed to 10 and 100 μM was significantly greater than that of controls (Fig. 2). However, exposure to 52 μM prometon did not result in significantly greater E2 production relative to controls, thus the concentration

response between 10 and 100 μM was not monotonic in the ovary explant assay (Fig. 2). In the H295R assay, prometon exposure similarly had no effect on T production while E2 production by cells exposed to 10 and 100 μM was significantly greater than that of control cells (Fig. 3). However, since a concentration intermediate between 10 and 100 μM was not tested in the H295R assay, it was unclear whether a similar non-monotonic profile would hold in both assays. Prometon concentrations, as great as 100 μM , were not toxic to the H295R cells. Overall, the two assays showed similar sensitivity to the effects of prometon.

The mean coefficient of variation (CV) for E2 production among replicate fathead minnow ovary explants exposed to prometon was greater than that observed for the other five chemicals tested (Table 1). Correspondingly, mean E2 production was generally skewed from the median value (greater) for prometon treated explants

Table 1
Mean (\pm SD) coefficients of variation (CVs)^a for relative estradiol and testosterone production responses among replicate wells of cells or replicate explants in the H295R and fathead minnow (FHM) ovary explant in vitro steroidogenesis assays, respectively, for each chemical tested

Chemical	H295R CVs (%)		FHM CVs (%)	
	Estradiol	Testosterone	Estradiol	Testosterone
Fadrozole	31.0 \pm 14.4	33.1 \pm 16.3	55.5 \pm 17.6	44.6 \pm 10.9
Fenarimol	14.7 \pm 8.9	19.1 \pm 13.5	27.0 \pm 23.0	12.4 \pm 6.8
Ketoconazole	12.8 \pm 13.6	23.9 \pm 24.7	22.3 \pm 11.5	37.2 \pm 20.2
Prochloraz	22.0 \pm 5.9	27.1 \pm 9.3	30.2 \pm 15.9	18.7 \pm 13.4
Prometon	11.3 \pm 9.8	27.1 \pm 21.8	89.1 \pm 37.1	40.7 \pm 7.2
Vinclozolin	13.6 \pm 7.2	19.5 \pm 16.8	45.9 \pm 13.4	37.1 \pm 16.7
ELISA/RIA ^b	10 \pm 7 ^c	14 \pm 9 ^c	13 \pm 11	10 \pm 9

^aMean and standard deviation of CV values calculated for each individual treatment group (H295R $n = 7$ groups for all chemicals except vinclozolin [$n = 10$]; FHM $n = 6$ groups for all chemicals except prometon [$n = 9$]). The CV value for each treatment group was calculated based on mean and SD across all replicate explants or wells after normalization to % control or % SC values, in order to account for variations among different batches of cells or different fish. Comparisons in the text were based on absolute differences among means and do not necessarily indicate statistically significant differences.

^bWithin assay CV of the enzyme-linked immunosorbent assay (ELISA; H295R) or radioimmunoassay (RIA; fathead minnow) methods used to quantify estradiol and testosterone. CV includes variability resulting from differences in extraction efficiency among samples. Does not include biological variability among replicate wells of cells or replicate ovary explants.

^cResults from Hecker et al. (2006).

(Fig. 2). This was not the case in the H295R assay. Instead E2 production among replicate wells of H295R cells was less variable (based on average CV) than that observed for the other five chemicals (Table 1). The mean CVs for T production by prometon treated cells and ovary explants was not notably different than those observed for the other chemicals (Table 1).

3.6. Vinclozolin

Direct comparison of H295R and fathead minnow ovary explant assay responses to vinclozolin was limited by the relatively small range of concentrations (0.1–1.0 μ M) tested in both systems. Nonetheless some differences were apparent, particularly for T production. The concentration–response curve for vinclozolin’s effect on T production by fathead minnow ovary explants was non-monotonic (Fig. 2). Exposure to 0.117 μ M vinclozolin caused a significant decrease in T production, while 1.05 μ M vinclozolin significantly increased ovarian T production (Fig. 2). Estradiol production by the ovary explants was not significantly affected by vinclozolin concentrations as great as 1.05 μ M (Fig. 2). Estradiol production by the H295R cells showed a weak but significant increase, but only following exposure to 100 or 300 μ M vinclozolin (Fig. 3; Hecker et al., 2006), concen-

trations much greater than those tested in the fathead minnow ovary explant assay. Testosterone production by H295R cells was significantly decreased after exposure to 100 or 300 μ M vinclozolin, and the concentration–response was monotonic (Fig. 3; Hecker et al., 2006). Vinclozolin concentrations $\geq 300 \mu$ M were found to be moderately toxic to the H295R cells (approximately 25% reduction in the % live cells). Exposure to vinclozolin concentrations around 0.1 and 1.0 μ M, reduced and increased T production by ovary explants, relative to controls, did not significantly alter T production by the H295R cells (Fig. 3). Overall, the fathead minnow ovary explant assay was more sensitive to effects of vinclozolin on T production, while the relative sensitivity to vinclozolin’s effects on E2 production could not be determined.

4. Discussion

4.1. Fadrozole

Fadrozole is a reversible, competitive, inhibitor of aromatase (CYP19, Fig. 1; Steele et al., 1987; Schieweck et al., 1988). It has been reported to inhibit various enzymes involved in aldosterone synthesis (Fig. 1), but only at concentrations 4–5 orders of magnitude greater than those needed to inhibit aromatase (Bhatnagar et al., 1990; Santen et al., 1990). Therefore, we hypothesized that H295R cells or fathead minnow ovary explants exposed to fadrozole would produce less E2 than control cells, while T production would be similar to or slightly greater than that of controls due to reduced conversion of T to E2 by aromatase and, in the case of H295R cells, possibly reduced flux of precursors like progesterone toward the aldosterone pathways (Fig. 1). Results from the fathead minnow ovary explant assay, as well as those from most concentrations tested in the H295R assay, supported this hypothesis. However, exposure to 100 μ M fadrozole significantly reduced T production by H295R cells. Exposure to similar concentrations did not cause ovary explants to produce less T, relative to controls, but the ovary explants were two orders of magnitude less sensitive to the effects of fadrozole on E2 production than were the H295R cells. From the data generated for this study, it is not possible to determine whether the difference in sensitivity to fadrozole was related to species differences, tissue specific differences (e.g., cross-talk with the corticosteroid pathways in the H295R cells), differences in the time course of the exposures (i.e., 48 h for the H295R versus 14.5 h for the ovary explant assay), or some other factor. Nonetheless, a concentration on the order of 10 mM might be required to see the effect in the ovary explant assay, due to the overall difference in sensitivity. We are not aware of any reports that suggest fadrozole can inhibit enzymes involved in T biosynthesis at concentrations just 2–3 orders of magnitude greater than those that inhibit aromatase activity. Hence, it is not clear why fadrozole reduced T production by the H295R cells or whether

greater concentrations (or longer exposures) could similarly reduce T production by fathead minnow ovary explants.

In vivo effects of fadrozole were consistent with inhibition of E2 synthesis, but not T synthesis. Waterborne exposure to 50 µg fadrozole/L (0.22 µM) for 21 d significantly inhibited brain aromatase activity in the fathead minnow (Ankley et al., 2002). Plasma E2 levels were significantly reduced in females exposed to ≥10 µg fadrozole/L (0.045 µM), while plasma androgen levels and gonadal-somatic index were significantly elevated in males exposed to the same concentrations (Ankley et al., 2002). Therefore, in terms of predicting potential effects of the chemical, the ovary explant assay showed better agreement with the in vivo data than the H295R results. However, from a screening perspective, the H295R was a more sensitive assay for detecting fadrozole's overall potential to affect steroidogenesis.

4.2. Fenarimol

Fenarimol is a chlorinated aromatic heterocyclic amine fungicide widely used in the production of fruits, vegetables, and ornamental plants. It has been reported to act as an estrogen, anti-androgen, anti-estrogen, and aromatase inhibitor, based on various in vitro and in vivo studies (see Andersen et al., 2006; Ankley et al., 2005). In the fathead minnow ovary explant assay, fenarimol caused a significant, concentration-dependent, decrease in T production, as well as a significant decrease in E2 production. The sensitivity of fathead minnow ovary explants to fenarimol's effect on E2 production was consistent with the sensitivity of fathead minnow ovary homogenates to inhibition of aromatase enzyme activity following in vitro exposure to fenarimol (Ankley et al., 2005). However, fenarimol's effects on both aromatase activity and E2 production in vitro was over 10-fold less potent than its effect on T production in vitro. Based on the assumption that the relatively short time-course (i.e., 14.5 h) of exposure and isolation of the ovary explant from the rest of the hypothalamic–pituitary–gonadal axis precluded any significant compensatory response to the estrogenic and/or anti-androgenic effects of fenarimol, results of the ovary explant assay suggest that fenarimol can potentially inhibit steroidogenic enzymes upstream of aromatase (CYP19; Fig. 1). Furthermore, given that E2 production was decreased only at the greatest concentration tested, the results suggest that potential upstream targets (e.g., CYP17, CYP11; Fig. 1) could have been more sensitive to fenarimol than aromatase.

Effects of fenarimol on E2 and T production by the H295R cells were different from those observed in the fathead minnow ovary explant assay. In particular, for the H295R assay, E2 production was far more sensitive (4 orders of magnitude) to fenarimol than was the production of T, whereas in the fathead minnow assay T production was more sensitive to fenarimol than E2

production. The IC₅₀ (concentration needed to cause 50% inhibition) for inhibition of aromatase activity in H295R cells was reported to be 80 µM fenarimol (Sanderson et al., 2002). Although the same cell line was used, the duration of exposure (24 h versus 48 h) and incubation conditions were slightly different. Nonetheless, given that fenarimol was 1000–10,000 times more potent at reducing E2 production by the H295R cells than it was in its effect on aromatase activity in the same cell line, it is likely that aromatase inhibition alone does not explain the observed reduction of steroid synthesis. Results of other studies with the H295R cells suggest that the H295R cell bioassay may have some capacity to mount autoregulatory (compensatory) responses to chemicals over the course of a 48 h exposure (Hecker et al., unpublished data). The greater sensitivity of the E2 response, relative lack of concentration dependence over a range from 0.01 to 3.0 µM, and step-like decrease in E2 production between 3.0 and 10 µM could plausibly indicate some type of autoregulatory response in the cells, coupled with aromatase inhibition and/or cytotoxicity at relatively great concentrations (i.e., >10 µM). A more detailed characterization of gene expression and hormone production by the cells over time would be needed to fully evaluate this possibility.

In the case of fenarimol, the overall pattern of effects in vivo was not readily predicted or explained by either of the in vitro steroidogenesis assays. In vivo exposure to 1.0 mg fenarimol/L (3.0 µM) for 21 d completely inhibited fathead minnow spawning (Ankley et al., 2005). Plasma E2 concentrations were significantly elevated in females exposed to the same concentration, but no significant changes in plasma androgen levels were observed (Ankley et al., 2005). A significant and potent increase in E2 production, with a much less potent, or no, effect on T production was not observed in either in vitro assay. Thus, the in vitro steroidogenesis assays lend little mechanistic insight to the response in vivo, beyond a suggestion that the direct effect of fenarimol on steroidogenic enzyme activities may not be the dominant cause of its effects in vivo. Predictive utility aside though, from a screening perspective, the H295R assay was again the more sensitive assay for simply detecting the chemical's potential to affect steroidogenesis.

4.3. Ketoconazole

Ketoconazole is a pharmaceutical fungicide that can act as a reversible inhibitor of a wide range of CYPs, including xenobiotic and steroid metabolizing enzymes (e.g., CYP1A1, CYP3A; Miranda et al., 1998; Hegelund et al., 2004) as well as CYPs involved in steroid synthesis (e.g., CYP11A, CYP17, and CYP19; Fig. 1; Kan et al., 1985; Weber et al., 1991). Ketoconazole caused significant, concentration-dependent, reduction in both E2 and T production in both assays and was a more potent suppressor of T production than E2 production. These results were consistent with previous reports that the effect

of ketoconazole on the C17,20-lyase (desmolase) activity of CYP17 was more potent than its effect on aromatase (e.g., Weber et al., 1991). Additionally, in previous experiments with fathead minnow ovary homogenates, the lowest observed effect concentration (LOEC) for ketoconazole's inhibition of aromatase activity was $1.3\ \mu\text{M}$ (Villeneuve et al., 2005) while T production by ovary explants was reduced by concentrations less than $0.01\ \mu\text{M}$. Consequently, the evidence suggests that ketoconazole is a more potent inhibitor of one or more upstream steroidogenesis enzymes than it is an inhibitor of aromatase.

Effects *in vivo* were not readily predicted by the concentration-dependent decreases in both E2 and T production observed in the H295R and fathead minnow ovary explant assays. *In vivo* exposure to ketoconazole did not cause significant decreases in plasma concentrations of E2 and T as one might have expected. However, rates of *ex vivo* steroid synthesis by ovary and testis tissue collected from the fish exposed to ketoconazole concentrations $\geq 25\ \mu\text{g/L}$ ($0.047\ \mu\text{M}$) *in vivo* were significantly lower than those of controls suggesting that steroidogenesis was being impacted, even though plasma E2 and T concentrations remained near control levels (Ankley et al., 2007). The apparent disconnect between the consistent effects of ketoconazole on *in vitro* (both assays) and *ex vivo* steroid production (i.e., *in vitro* hormone production by tissue collected from fish exposed *in vivo*), relative to a lack of effects on plasma steroid concentrations, suggests that *in vivo* compensatory mechanisms (e.g., proliferation of steroidogenic cells) were able to offset some of the direct effects of ketoconazole on steroidogenic enzyme activities in exposed fish (Ankley et al., 2007). Nonetheless, reproductive success was still reduced in fish exposed to ketoconazole concentrations as low as $25\ \mu\text{g/L}$ (Ankley et al., 2007). In terms of Tier I screening, the fathead minnow ovary explant assay detected ketoconazole's effect on steroidogenesis at concentrations lower than those that adversely affected reproduction (e.g., $0.047\ \mu\text{M}$), but the H295R assay did not. Effect concentrations in the H295R assay were over 20-fold greater than the concentration that adversely affected fathead minnow reproduction. Thus, in the case of ketoconazole the ovary explant assay performed more favorably from a screening perspective, while neither assay was readily predictive of effects *in vivo*.

4.4. Prochloraz

Prochloraz is a widely used agricultural imidazole fungicide. Like other imidazole fungicides, it inhibits a CYP enzyme involved in ergosterol synthesis, thereby inhibiting fungal growth (Henry and Sisler, 1984), but it can also induce or inhibit different CYPs in fish and mammals (Laignelet et al., 1989; Snegaroff and Bach, 1989). Prochloraz caused similar responses in both *in vitro* steroidogenesis assays. There were concentration-dependent decreases in both E2 and T production and the effect on E2 production was more potent than the effect on T.

The concentration-dependent decrease in T production suggests that prochloraz inhibited one or more steroidogenic enzymes upstream of aromatase (Fig. 1). However, the fact that prochloraz was a more potent suppressor of E2 production than T production indicates that the aromatase enzyme was likely more sensitive to inhibition by prochloraz than the upstream targets (Fig. 1).

The effects prochloraz on both androgen synthesis and estrogen synthesis detected *in vitro* were also evident *in vivo*. *In vivo* exposure to $300\ \mu\text{g prochloraz/L}$ ($0.80\ \mu\text{M}$) for 21 d significantly impacted plasma steroid concentrations in exposed fathead minnows (Ankley et al., 2005). Androgen levels (T and 11-ketotestosterone) were significantly decreased in males, while in females E2, but not androgen, concentrations were less than those in controls (Ankley et al., 2005). Thus, in the case of prochloraz, both *in vitro* assays were reasonably predictive of effects *in vivo*, although the difference in response between sexes would not necessarily be deduced from *in vitro* results alone.

Prochloraz concentrations $\geq 100\ \mu\text{g/L}$ ($0.27\ \mu\text{M}$) significantly reduced fathead minnow fecundity (Ankley et al., 2005). The H295R assay detected a significant effect on steroidogenesis at concentrations as low as $0.03\ \mu\text{M}$, around 10-fold less than the concentration that impacted fecundity *in vivo*. In contrast, the LOEC in the fathead minnow ovary explant assay was $3\ \mu\text{M}$, about 10-fold greater than the LOEC for effects on fecundity. Thus, from a screening standpoint, the H295R assay was more sensitive than the fathead minnow ovary explant assay in detecting the potential effect of prochloraz.

4.5. Prometon

Prometon is a non-selective methoxytriazine herbicide widely used for bare-ground weed control around buildings, fences, railroads, recreational areas, and rights-of-way (<http://www.pramitol.com>). Despite the fact that it is not widely registered for agricultural, home, or garden use, prometon has been commonly detected in environmental samples (Bruce and McMahon, 1996; Adamski and Pugh, 1996; Kolpin et al., 1998; Larson et al., 1999; Hoffman et al., 2000). Based on the concentrations detected in the environment, concentrations on the order of $0.25\ \mu\text{g/L}$ ($0.001\ \mu\text{M}$) might be expected in many North American surface and groundwaters, while approximately $5\ \mu\text{g/L}$ ($0.022\ \mu\text{M}$) represents the upper range of environmentally relevant concentrations for urban streams, and concentrations around $80\ \mu\text{g/L}$ ($0.355\ \mu\text{M}$) could be considered an upper range for groundwater concentrations (Bruce and McMahon, 1996; Adamski and Pugh, 1996; Kolpin et al., 1998; Larson et al., 1999; Hoffman et al., 2000). In the H295R and fathead minnow ovary explant assays, prometon concentrations $\geq 10\ \mu\text{M}$ significantly enhanced E2 production *in vitro*. Even factoring in a bioconcentration factor as great as eight in fish (Villeneuve et al., 2006), the concentrations that affected E2 production *in vitro* were at least 4-fold greater than the upper bound of

reported groundwater concentrations and around 130-fold greater than the upper range of concentrations measured in urban streams.

Neither of the *in vitro* assays provided results that could be considered readily predictive of effects observed *in vivo* after 21 d of exposure to prometon. *In vivo* exposure to concentrations of 20, 200, and 1000 µg/L (0.089, 0.89, and 4.4 µM) significantly decreased fatpad index in males (Villeneuve et al., 2006). This could be considered a demasculinizing effect which one could suggest might be related to elevated estrogen production *in vivo*, but plasma steroid concentrations did not support such a connection. Plasma E2 concentrations *in vivo* were unaffected by prometon exposure, while plasma T was increased in females exposed to 20 µg/L (although not other concentrations of prometon; Villeneuve et al., 2006). *In vivo*, concentration–response relationships for prometon were non-monotonic. The possibility that the non-monotonic response observed in the fathead minnow ovary explant assay was reflective of a complex, indirect mechanism of action both *in vitro* and *in vivo*, remains to be tested.

From the standpoint of a potential screening assay, the H295R assay had slight advantages over the fathead minnow ovary explant assay relative to detecting effects of prometon. Sensitivity of the two assays to prometon was the same. However, the responses of the H295R assay to the herbicide were much less variable than that of fathead minnow ovary explants, particularly for E2 production (Table 1). Although in general mean CVs among replicate wells of H295R cells were less than those among replicate fathead minnow ovary explants, the difference was particularly pronounced for prometon. It was not clear from the data whether this reflected a mechanistic difference between the two systems or whether it was random.

4.6. Vinclozolin

Vinclozolin is a dicarboximide fungicide widely used to control fungal growth on fruits, vegetables, ornamental plants, and turf-grass (US EPA, 2000). The active metabolites of vinclozolin, M1 (2-[[[(3,5 dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenic acid) and M2 (3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide) act as androgen receptor (AR) antagonists (Gray et al., 1994; Kelce et al., 1994). In previous experiments with H295R cells, vinclozolin was able to induce aromatase activity at concentrations exceeding 10 µM, likely by increasing cAMP levels (Sanderson et al., 2002). Given the unknown capacity of H295R cells or fathead minnow ovary explants to convert vinclozolin to M1 or M2 *in vitro*, and evidence for other indirect mechanisms (e.g., increased cAMP), it was unclear what effects, if any, vinclozolin would have in the *in vitro* steroidogenesis assays.

Comparison of the H295R and fathead minnow ovary explant assay results did little to clarify the mechanistic

basis for vinclozolin's effects on steroid production. It has been suggested that the slight up-regulation in E2 production (Fig. 3), concomitant with decreases in T (Fig. 3) and pregnenolone/progesterone production (see Hecker et al., 2006) at concentrations ≥ 100 µM that was observed, in the H295R assay could be attributed to a selective, cAMP-mediated upregulation of aromatase activity in the cells, along with the resulting greater flux of progesterone and androgenic precursors toward estrogen production (Fig. 1; Hecker et al., 2006). However, feedback mechanisms related to antagonism of the AR by vinclozolin metabolites (if present) might also have influenced the responses (Hecker et al., 2006). The non-monotonic concentration response in the fathead minnow ovary explant assay (i.e., 0.117 µM caused a significant decrease in T production while 1.05 µM significantly increased T production) also suggested a complex indirect mechanism of action for vinclozolin's effects on steroid production. Additional understanding of the metabolic capabilities of the H295R cells and fathead minnow ovary explants, and the role of the AR in regulating or cross-talking with steroidogenic pathways are needed to fully explain the response profiles observed. What was clear was that vinclozolin influenced steroid production *in vitro*, and effects in the two *in vitro* steroidogenesis assays differed substantially.

Following 21 d of *in vivo* exposure to vinclozolin, ovary explants from females exposed to ≥ 100 µg/L (0.35 µM) were producing 4–5-fold greater T concentrations than those from control females but E2 production was unaffected (Martinovic et al., 2006). Thus, the *in vitro* steroidogenesis assay using fathead minnow ovary explants yielded results that were fairly consistent with those induced in gonad tissue from fish exposed *in vivo* (*ex vivo* steroidogenesis). The H295R results were not predictive of these effects.

In a recent 21 d study with vinclozolin from our lab, concentrations ≥ 100 µg/L caused a concentration-dependent reduction in fathead minnow fecundity (Martinovic et al., 2006). From a screening perspective, the fathead minnow ovary explant assay would have detected some activity at a lower concentration (i.e., 0.1 µM). The LOEC in the H295R assay was 100 µM, a concentration 250 times greater than the concentrations that adversely affected fathead minnow fecundity. However, it is notable that, given vinclozolin's mechanism of action, one would expect a Tier I screening assay for anti-androgens (e.g., the Hershberger assay; EDSTAC, 1998) to flag vinclozolin for further testing even if it failed to produce a response in the H295R cells.

4.7. Assay evaluation

Measurements of E2 and T production by H295R cells or fathead minnow ovary explants were useful for identifying chemicals as potential EACs. Based on the responses to the six chemicals evaluated in this study, neither assay was universally more sensitive. The H295R

assay was more sensitive for detecting effects of fadrozole, fenarimol, and prochloraz, but was less sensitive than fathead minnow ovary explants to the effects of ketoconazole and vinclozolin. It is notable that in the fathead minnow ovary explant assay, fadrozole, fenarimol, and prochloraz were delivered in methanol or ethanol, while ketoconazole and vinclozolin were delivered as aqueous stocks dissolved directly in supplemented culture medium. Consequently, some of the difference in sensitivity among assays may be reflective of differences in uptake efficiency associated with different carriers or suppression of responses by the solvents. However, mean E2 and T production by ovary explants exposed to methanol or ethanol alone did not differ from that of control explants. Further investigation would be required to determine whether carrier solvent significantly influenced the sensitivity of the ovary explant assay.

Comparing average CVs among replicate wells for each different chemical exposure, the H295R assay was generally less variable than the fathead minnow ovary explant assay (Table 1). This is not surprising given the heterogeneous nature of fathead minnow ovary tissue compared to cultured H295R cells. Beyond the fact that the ovary explants include multiple cell types, fathead minnows are asynchronous spawners with follicles of many different stages present in their ovaries. On a mass or volume basis, steroidogenic capacity of follicles can vary considerably with their relative stage of maturity (McMaster et al., 1995). Variability of the fathead minnow ovary explant assay could likely be reduced by sorting follicles by size and exposing a uniform number of same-sized follicles in each well; however, such an approach would be highly labor intensive, and generally not practical for moderate to high-throughput applications.

Results of this study indicate that responses in the H295R cell-based steroidogenesis assay are not necessarily directly predictive of the effects of a chemical on synthesis of reproductive steroids within fish gonad tissue. For at least two of the chemicals tested (fadrozole and fenarimol), the profile of responses differed markedly between the two assays. These differences reflected not only differences in sensitivity, but more fundamental variation in the likely mechanism(s) underlying the effects. Additionally, in the H295R assay, T responses tended to be more variable among replicate wells than E2 responses, while the opposite was true for the fathead minnow ovary explant assay (Table 1). While some of these differences may be attributed to differences in the complexity of the two systems (e.g., tissue versus cells), tissue- and species-related differences should also be considered. The dominant steroidogenic pathways in adrenal tissue differ from those in gonad tissue (Fig. 1; Norris, 1997). Additionally, it has been hypothesized that fish and mammalian steroidogenic enzymes (e.g., HSDs, CYP19, and CYP hydroxylases) may differ in specificity for some chemicals (Baker, 2001). Furthermore, sex steroids play different roles in oviparous (e.g., fish) versus viviparous animals (e.g., rodents) (Norris,

1997). Consequently, some steroidogenic processes are likely to be regulated differently between fish and mammals, which could also result in different sensitivities and/or responses to EACs. As a result, although the H295R assay may be suitable in a Tier I screening role, at present, extrapolation of H295R assay results to understand mechanism of action and predict effects in other biological systems (e.g., different tissues and different species) would likely involve considerable uncertainty.

For the chemicals examined in this study, results of the fathead minnow ovary explant assay were generally more closely aligned with results from 21d fathead minnow reproduction tests than those of the H295R assay. However, the ability to use either assay to link mechanism of action in vitro to apical responses in vivo was complicated by the fact that responses in both assays seemed to reflect more than just direct inhibition or potentiation of the activity of steroidogenic enzymes. The differences in the responses of the two assays, and observations like the non-monotonic effects of prometon and vinclozolin in the fathead minnow ovary explant assay, and increased cAMP (Sanderson et al., 2002) and changes in the expression of steroidogenic genes (Zhang et al., 2005; Hilscherova et al., 2004) in H295R cells exposed to EACs all suggest that E2 and T production in vitro can be affected in multiple ways. Detailed characterization of the test systems is needed if either H295R or fathead minnow ovary explant results are to provide reliable mechanistic insights, in the absence of a priori information regarding a chemical's mechanism of action. In particular, better characterization of autoregulatory processes involved in transcriptional regulation along the steroidogenic pathways, the functions of steroid hormone receptors within the cells or explants, and the metabolic capabilities of the cells would all help define the potential mechanisms through which in vitro E2 and T production could be modulated. Interpretation of assay results could also be aided by computational models that could predict likely shifts in the flux of various steroid precursors that would result from inhibition of one or more enzymes in the synthetic pathway (Fig. 1; e.g., Breen et al., 2007). Such modeling would be particularly useful for the H295R system in which interactions between the corticosteroid and reproductive steroid synthesis pathways might occur (Fig. 1).

5. Conclusions

The H295R and fathead minnow ovary explant assays both have utility for detecting EACs in screening-type applications. Although neither assay was consistently more sensitive, the lesser variability of the H295R assay, coupled with the ability to screen for a broader complement of steroid production without the use of animals, makes it more favorable than the ovary explant assay as a routine, high throughput, screening tool. Differences in the responses of the two assays, and the complex concentration–response relationships observed for some chemicals,

highlight the need for greater biological characterization and the complementary use of predictive computational models if either assay is to be used, with reasonable certainty, to define mechanism of action and/or predict in vivo effects in fish.

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