

Assessment of chemical effects on aromatase activity using the H295R cell line

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Abstract

Background, aim, and scope In response to concerns about chemical substances that can alter the function of endocrine systems and may result in adverse effects on human and ecosystem health, a number of in vitro tests have been developed to identify and assess the endocrine disrupting potential of chemicals and environmental samples. One endpoint that is frequently used in in vitro models for the assessment of chemical effects on the endocrine system is the alteration of aromatase activity (AA). Aromatase is the enzyme responsible for converting androgens to estrogens. Some commonly used aromatase assays, including the human microsomal assay that is a mandatory test in US-EPA's endocrine disruptor screening program (EDSP), detect only direct effects of chemicals on aromatase activity and not indirect effects, including changes in gene expression or transcription factors. This can be a problem

for chemical screening initiatives such as the EDSP because chemicals can affect aromatase both indirectly and directly. Here we compare direct, indirect, and combined measurements of AA using the H295R cell line after exposure to seven model chemicals. Furthermore, we compare the predictability of the different types of AA measurements for 17 β -estradiol (E2) and testosterone (T) production in vitro.

Materials and methods H295R cells were exposed to forskolin, atrazine, letrozole, prochloraz, ketoconazole, aminoglutethimide, and prometone for 48 h. Direct, indirect, and combined effects on aromatase activity were measured using a tritiated water-release assay. Direct effects on aromatase activity were assessed by exposing cells only during the conduct of the tritium-release assay. Indirect effects were measured after exposing cells for 48 h to test chemicals, and then measuring AA without further chemical

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addition. Combined AA was measured by exposing cells prior and during the conduction of the tritium-release assay. Estradiol and testosterone were measured by ELISA.

Results and discussion Exposure to the aromatase inhibitors letrozole, prochloraz, ketoconazole, and aminoglutethimide resulted in greater indirect aromatase activity after a 48-h exposure due to presumed compensatory mechanisms involved in aromatase activity regulation. Forskolin and atrazine caused similar changes in hormone production and enzyme profiles, and both chemicals resulted in a dose-dependent increase in E2, T, and indirect AA. Neither of these two chemicals directly affected AA. For most of the chemicals, direct and combined AA and E2 were good predictors of the mechanism of action of the chemical, with regard to AA. Indirect aromatase activity was a less precise predictor of effects at the hormone level because of presumed feedback loops that made it difficult to predict the chemicals' true effects, mostly seen with the aromatase inhibitors. Further, it was found that direct and indirect AA measurements were not reliable predictors of effects on E2 for general inducers and inhibitors, respectively.

Conclusions Differential modulation of AA and hormone production was observed in H295R cells after exposure to seven model chemicals, illustrating the importance of measuring multiple endpoints when describing mechanisms of action in vitro.

Recommendations and perspectives For future work with the H295R, it is recommended that a combination of direct and indirect aromatase measurements is used because it was best in predicting the effects of a chemical on E2 production and its mechanism of action. Further, it was shown that direct AA measurements, which are a common way to measure AA, must be used with caution in vitro.

Keywords Estradiol · Testosterone · Endocrine · Hormone · Forskolin · Atrazine · Letrozole · Prochloraz · Ketoconazole · Aminoglutethimide · Prometon · CYP19 · Steroidogenesis · Compensation

1 Introduction

Exposure to natural and man-made substances in the environment has been linked to alterations in endocrine and reproductive systems in wildlife (Ankley et al. 1998; Sumpter and Johnson 2005; Jobling et al. 2006). Some chemicals are receptor agonists and act directly as hormone mimics. One group of chemicals that has received increased attention during the past two decades are environmental (xeno)estrogens such as 17 β -estradiol (E2), ethinylestradiol, and other estrogen receptor agonists such bisphenol A

and some alkylphenolics. Other chemicals can modulate the endocrine system by acting through non-receptor mediated mechanisms. For instance, substances such as some imidazole-like fungicides and phyto-flavonoids have been shown to modulate hormone production by affecting activities of the steroidogenic enzymes aromatase (CYP 19) and 17 β -hydroxysteroid-dehydrogenase (HSD), respectively (Sanderson et al. 2001; Brooks and Thompson 2005).

One non-receptor-mediated pathway of endocrine disruption chemicals (EDCs) of concern is the interference with sex steroid synthesis, specifically the production of 17 β -estradiol, by the enzyme aromatase. Aromatase is a member of the cytochrome P450 family and catalyzes the conversion of testosterone to estradiol in various tissues of vertebrates. Disruption of aromatase can lead to significant alterations in the endocrine homeostasis in organisms. For example, male and female aromatase knock-out mice had decreased production of estradiol and elevated concentrations of testosterone. Furthermore, disruption of aromatase in these mice also impaired spermatogenesis and sexual behavior in male rats and resulted in severely under-developed uteri in female rats (Simpson et al. 2002). While the formation of estrogens via aromatase is of great importance in context with the development and reproductive physiology of vertebrates, it is also discussed in context with the role of estrogens as promoters of carcinogenesis (Ryan 1982).

The aromatase enzyme can be the target of some environmentally relevant chemicals and can affect production of E2 and testosterone (T) (Sanderson et al. 2001; Hecker et al. 2006). Chemicals can affect aromatase activity by reacting directly with the enzyme or through other indirect mechanisms. Direct interactions of a chemical with the aromatase enzyme can include competition of the EDC with the endogenous ligand or by interfering with important biochemical processes in the conversion of T to E2. In addition to direct effects of chemicals on aromatase activity that are typically of an inhibiting nature, indirect effects can result in either decreased or increased aromatase activity.

Concerns regarding the aromatase disrupting properties of certain chemicals have resulted in the inclusion of effects on aromatase activity in recent endocrine disruptor screening initiatives, such as the mandatory Endocrine Disruptor Screening Program (EDSP) of the US-EPA. The assay currently promoted by the EPA is the human recombinant microsomal aromatase assay, which measures direct effects on aromatase activity. This assay can be advantageous for measuring direct inhibitory effects of chemicals on aromatase activity but it does not allow detecting indirect effects that have been previously reported for a number of chemicals. This can be a problem with regard to detecting chemicals as disruptors of aromatase activity. For example, direct assays would not capture indirect effects due to the

induction or inhibition of CYP19 gene expression, e.g., through cAMP-mediated processes (Naville et al. 1999). Furthermore, EDCs can act through feedback mechanisms that can result in up- or down-regulation of aromatase activity while they do not necessarily interact directly with the enzyme. For example, if an EDC disrupts the metabolism of estradiol and results in changes in estradiol levels, aromatase activity might also be affected by an organism's attempt to maintain E2 homeostasis by regulating its production (Ung and Nager 2009).

Assays have been developed to evaluate the potential effects of chemicals on aromatase. Most of these assays, however, only measure a specific endpoint such as aromatase gene expression or aromatase enzyme activity, and it is unclear whether the observed changes are truly predictive of effects at the hormone level. One cell line that has been shown to be a useful *in vitro* model for steroidogenic pathways and processes, including production of sex steroids, and the aromatase enzyme is the human H295R adrenocarcinoma cell line (Sanderson et al. 2001; Hilscherova et al. 2004; Hecker et al. 2006, 2007). Interest in this assay as a screening tool is based on its unique ability to express all the steroidogenic hormones and enzymes and has been shown to be useful in screening for effects on gene expression of steroidogenic enzymes, steroidogenic enzyme activity, and production of steroid hormones (Gazdar et al. 1990; Rainey et al. 1993; Staels et al. 1993; Hilscherova et al. 2004; Gracia et al. 2006; Hecker et al. 2006; Sanderson 2006). Furthermore, under the guidance of the US-EPA and OECD, a H295R steroidogenesis assay has been developed to address regulatory needs for screening of the potential effects of chemicals on steroidogenesis pathways (Hecker and Giesy 2008).

The objective of the current study was to investigate the differential effects of selected model chemicals on different aromatase activity (AA) endpoints, namely direct, indirect, and combined AA measurements by exposing H295R cells to seven model chemicals with known interactions with the aromatase enzyme. This study further aimed to assess these differential AA measurement endpoints as predictors of changes of T and E2. Furthermore, multiple endpoints, including direct and indirect effects on aromatase activity and production of sex steroids, were used to develop a predictive classification scheme for these chemicals. The responses of the H295R cells to seven model EDCs were studied: letrozole, a specific aromatase inhibitor used in breast cancer treatment; prochloraz and ketoconazole, imidazole fungicides that have been shown to be aromatase inhibitors; forskolin, a cAMP inducer; atrazine and prometon, triazine herbicides and suspected endocrine disruptors that have previously been shown to induce aromatase activity and E2 production *in vitro*; aminoglutethimide, blocks p450 side-chain cleavage and inhibits aromatase.

2 Materials and methods

2.1 Test chemicals

Forskolin, ketoconazole, and aminoglutethimide were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Prochloraz was purchased from Aldrich (St. Louis, MO, USA). Letrozole was provided by Cstchem (Zhejiang) Co., Ltd. Prometon (technical grade, 98.7% purity, Lot 0310070) was obtained from Platte Chemical (Greenville, MS, USA), and atrazine (CAS number 1912-24-9; purity 97.1%) was obtained from Syngenta Crop Protection Inc. (Greensboro, NC, USA).

2.2 Cell culture

The H295R human adrenocortical carcinoma cell line was purchased from the American Type Culture Collection (ATCC CRL-2128; ATCC, Manassas, VA, USA) and grown as described previously (Hilscherova et al. 2004). Cells were cultured in 100-mm² petri dishes with 12.5 mL of supplemented medium at 37°C with a 5% CO₂ atmosphere. Briefly, the cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (Sigma D-2906; Sigma, St. Louis, MO, USA) supplemented with 1.2 g/L Na₂CO₃, 10 mL/L ITS + Premix (BD Bioscience; 354352), and 25 mL/L of BD Nu-Serum (BD Bioscience; 355100) unless specified differently.

2.3 Experimental design

All experiments were conducted in 24-well cell culture plates (COSTAR, Bucks, UK) with a cell concentration of 300,000 cells/mL. One mL of cell suspension 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 to test chemicals for 48 h. Dimethyl sulfoxide (DMSO) was used as carrier solvent and did not exceed 0.1% *v/v*. Test plates included six chemical concentrations and a solvent control (SC), in duplicate or triplicate. At the end of each experiment, the culture medium was transferred to an Eppendorf tube and stored at -80°C prior to analysis for hormones, and the live cells were subjected to the tritiated water-release assay for determination of aromatase activity.

To identify whether test chemicals could directly interact with the aromatase enzyme activity, a second series of experiments was conducted. For these experiments, a subset of three chemical concentrations was selected based on their activity observed during the above-described experiments, reflecting low, medium, and high responses of aromatase activity or changes in hormone production

(whichever applicable). Each chemical exposure experiment was conducted in three different ways: (1) *indirect aromatase activity*—cells were exposed in duplicate to each concentration of a chemical or SC for 48 h, and all chemical was rinsed and removed with phosphate-buffered saline (PBS), and aromatase activity was measured as described below; (2) *combined aromatase activity*—cells were exposed as described in the indirect assay, and the same chemical concentrations were added again during the conduct of the tritium-release assay to evaluate combined effects of pre-exposure and direct interaction with catalytic enzyme activity; and (3) *direct aromatase activity*—chemicals were added to untreated cells during the conduct of the tritium-release assay at the same concentrations as described in the combined aromatase assay to assess their direct interaction with the enzyme.

Prior to exposure, cell viability was evaluated in the SC and the three greatest exposure test chemical concentrations of each chemical with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) bioassay (Mosman 1983). Cytotoxic chemical concentrations were not included in the data evaluation.

2.4 Aromatase activity measurements

Aromatase enzyme activity was measured using a tritiated water-release assay as described by Lephart and Simpson (1991) with minor modification (Sanderson et al. 2001). After the H295R cells were exposed for 48 h, they were washed twice with 500 μ L PBS, and then 0.25 mL of supplemented medium containing 54 nM 1β - 3 [H]-androstenedione (Perkin Elmer, Boston, MA, USA) was added to each well. It is important to note that while most of the chemical was removed during the washing process, some of the chemical might still be present within the cells. For the experiments in which direct effects of chemicals on catalytic enzyme activity were measured, the chemical of interest was added at the appropriate concentration to the medium containing 1β - 3 [H]-androstenedione. DMSO was used as carrier solvent and did not exceed 0.1% *v/v*. The cells were then placed in an incubator at 37°C and 5% CO₂ for 1.5 h. After 1.5 h, cells were placed on ice to stop the reaction. A 200- μ l aliquot of the medium was removed and added to chloroform and Dextran-coated charcoal to remove all remaining 1β - 3 H-androstenedione. Aromatase activity was determined by the rate of conversion of 1β - 3 H-androstenedione to estrone by aromatase. The quantity of 3 H in extracts of medium was determined by liquid scintillation counting. Aromatase activity was expressed as picomoles of androstenedione converted per hour per 100,000 cells. The specificity of the reaction for the substrate was determined by use of a competitive test with non-labeled 1β -androstenedione and the use of the specific

aromatase inhibitor fadrozole (Hecker et al. 2005). Addition of large amounts of 1β -androstenedione reduced tritiated water formation to the concentrations found in the blanks. Furthermore, addition of fadrozole during the tritium-release assay reduced aromatase enzyme activity in a dose-dependent manner with concentrations of 0.3 μ M and greater resulting in complete inhibition of enzyme activity to the levels measured in the blanks.

2.5 Quantification of hormones

Frozen medium from exposures was thawed on ice, and hormones were extracted twice with diethyl ether (5 mL) in glass tubes, and phase separation was achieved by centrifugation at 2,000 \times g for 10 min. The solvent was evaporated under a stream of nitrogen, and the residue was dissolved in ELISA assay buffer and was either immediately measured or frozen at -80°C for later analysis. Hormones in culture medium were measured by competitive ELISA using the manufacturers' recommendations (Cayman Chemical Company, Ann Arbor, MI, USA; testosterone [Cat no. 582701], 17β -estradiol [Cat no. 582251]). Extracts of culture medium were diluted 1:2, 1:5, 1:10, 1:50, or 1:100 for estradiol and 1:50, 1:100, 1:150, 1:250, 1:500, 1:1,000, or 1:2,000 for testosterone prior to use in the ELISA.

2.6 Statistical analyses

Statistical analyses of hormone data were conducted using SYSTAT 11 (SYSTAT Software Inc., Point Richmond, CA, USA). All dose-response data were analyzed for significant differences using Kruskal–Wallis one-way analysis of variance. The Mann–Whitney *U* test was then performed to analyze differences of single doses from controls. The Pearson correlation test with the Bonferroni test was used to evaluate the associations between E2, T, and aromatase activity. The Pearson correlation test was also used to test for correlations between all endpoints when the data for all seven chemicals were combined. Differences with *p*<0.05 were considered to be statistically significant.

3 Results

3.1 General inducers

3.1.1 Atrazine

Atrazine significantly induced indirect and combined aromatase activity but direct aromatase activity was not statistically different from control levels at any concentration tested. Aromatase activity measured in the indirect and

combined aromatase assays was approximately 2.0-fold greater than control levels (Fig. 1 A). However, the increase in the combined assay was less pronounced than that observed in the indirect assay and was significantly different compared to the solvent control starting at 10 μM. Atrazine significantly increased E2 production in a dose-dependent manner at concentrations ≥1 μM. E2 concentrations were approximately 7.2-fold greater than those of the unexposed (SC) cells for the greatest dose tested (Fig. 1 A). Testosterone was significantly increased at the two greatest concentrations of 10 and 100 μM but the magnitude of the change was less than observed for E2 (maximum fold change of E2=7.2; maximum fold change of T=1.4). Statistically significant, positive correlations were observed between E2 and T ($r=0.944;p=0.004$) and

between the hormones and indirect aromatase activity (T: $r=0.882; p\leq 0.026$; E2: $r=0.952; p<0.003$).

3.1.2 Forskolin

Forskolin increased indirect and combined aromatase activity, E2, and T. Indirect aromatase activity was increased in dose-dependent manner up to 16-fold relative the solvent controls when exposed to forskolin in the H295R cells (Fig. 1 B). Direct aromatase activity showed no change when forskolin was added directly to the tritium-release assay for cells that had not been pre-exposed. In the combined aromatase assay, aromatase activity was 9.4-fold greater than that of unexposed cells. This increase was less than that measured in the indirect aromatase activity assay.

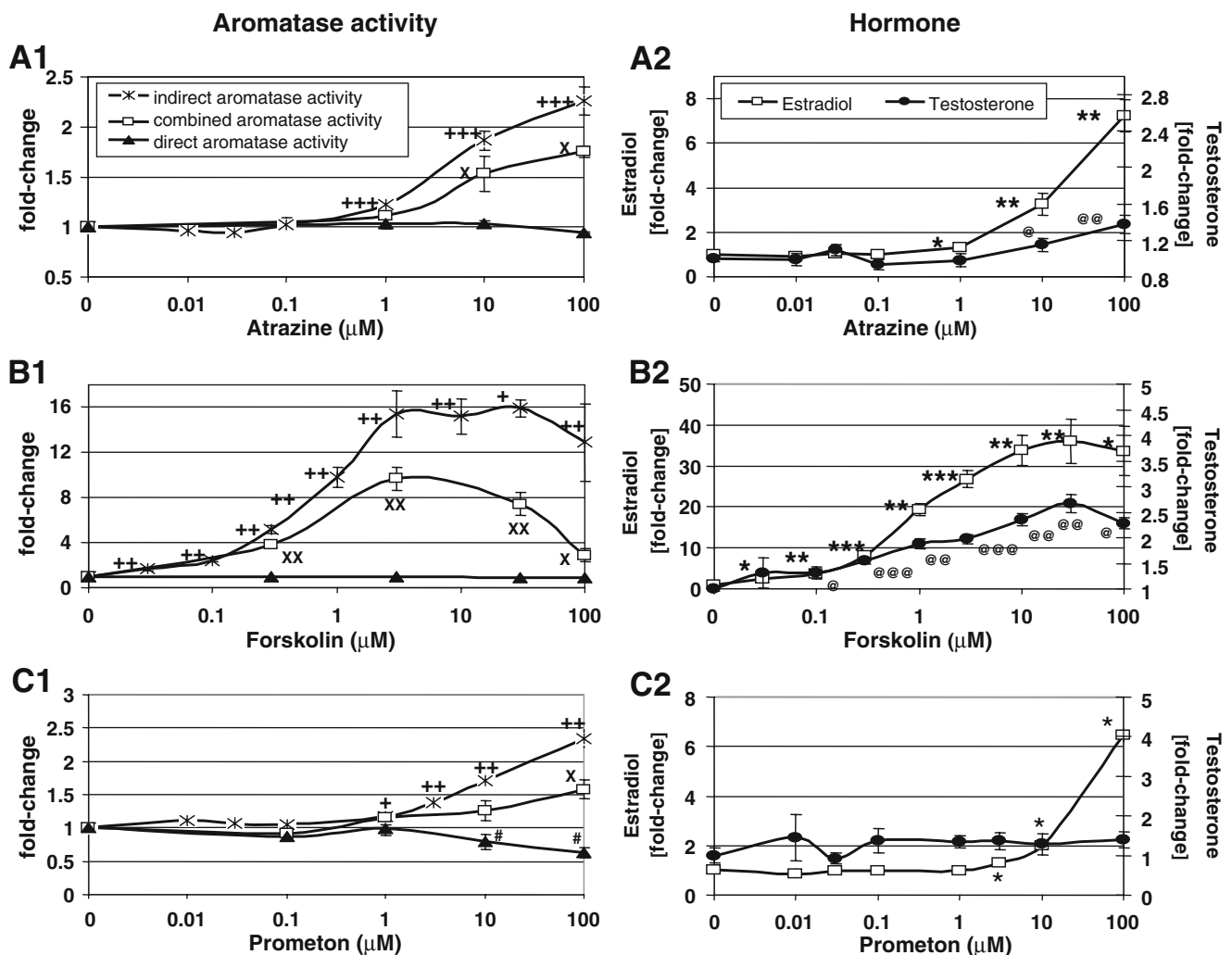


Fig. 1 General inducers: effects of the exposure of H295R cells with forskolin, atrazine, and prometon on testosterone, estradiol, and direct and indirect aromatase activities. Cells were treated for 48 h with the indicated concentrations of forskolin, atrazine, and prometon. Hormone data and aromatase activity are expressed as fold changes compared to solvent controls (SC=1). Values represent the mean ±

SEM. Significant differences for estradiol (asterisks), testosterone (at symbols), and indirect (plus signs) and direct (number signs) aromatase activity are reported relative to the solvent control. Multiple symbols indicate different significant levels: one symbol = $p<0.05$; two symbols = $p<0.01$; three symbols = $p<0.001$

Forskolin caused a statistically significant dose-dependent increase in production of E2 and T (Fig. 1 B). E2 production increased by 36-fold, while T increased by 2.7-fold relative to solvent controls. Statistically significant, positive correlations were observed between E2 and T ($r=0.899$; $p<0.001$) and the hormones and indirect aromatase activity (T: $r=0.899$; $p<0.001$; E2: $r=0.976$; $p<0.001$).

3.1.3 Prometon

E2 production and indirect aromatase activity were significantly greater in cells exposed to prometon at $\geq 3\mu\text{M}$ relative to controls. In the direct aromatase assay, activity was significantly less than that in the SCs at the two greatest doses tested (10 and 100 μM). No effect on the production of T was observed at any prometon concentration (Fig. 1 C). Correlation between concen-

trations of E2 and indirect aromatase activity levels were statistically significant and positive ($r=0.853$; $p<0.001$). No statistically significant correlation was observed between E2 and T ($r=0.025$; $p=1.00$).

3.2 Inhibitors

3.2.1 Letrozole

Exposure to letrozole resulted in statistically significant, dose-dependent reduction in E2 and T production as well as a reduction in direct and combined aromatase activities (Fig. 2 A). In the indirect aromatase assay, enzyme activity was significantly greater than in the controls at concentrations between 0.001 and 0.1 μM , while a decrease was observed at concentrations greater than 1 μM . Concentrations of E2 were significantly less

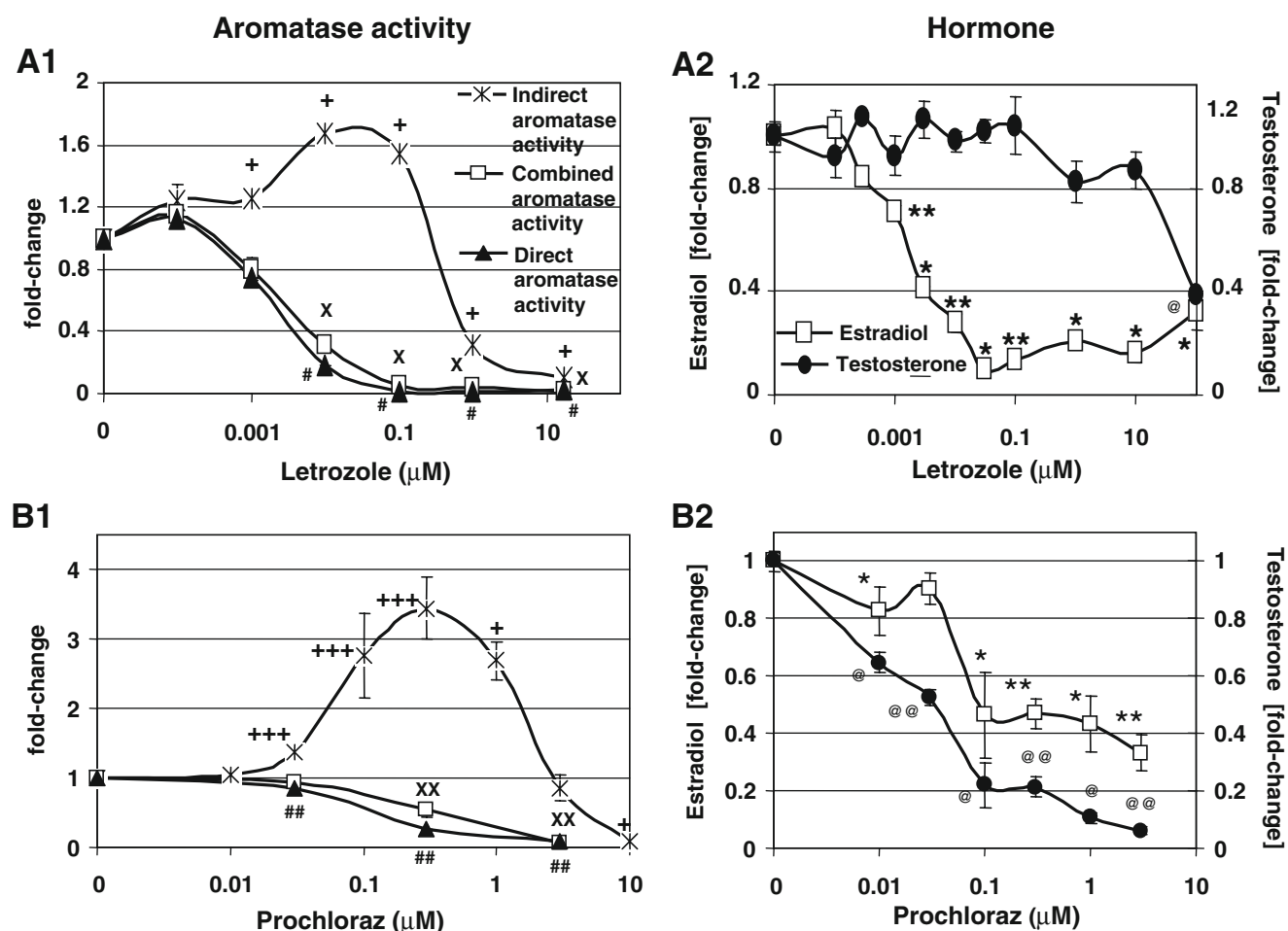


Fig. 2 Potent aromatase inhibitors: effect of letrozole and prochloraz on testosterone, estradiol, and direct and indirect aromatase activity by H295R cells. Cells were treated for 48 h with the indicated concentrations of letrozole and prochloraz. Hormone data are expressed as fold changes compared to solvent controls (SC= 1). Values represent the mean \pm SEM. Significant differences for

estradiol (asterisks), testosterone (at symbols), and indirect (plus signs) and direct (number signs) aromatase activity are reported relative to the solvent control. Multiple symbols indicate different significant levels: one symbol = $p<0.05$; two symbols = $p<0.01$; three symbols = $p<0.001$

than SC levels at concentrations $\geq 0.001 \mu\text{M}$ letrozole. A statistically significant decrease in T production was measured in the $100 \mu\text{M}$ letrozole exposure.

3.2.2 Prochloraz

Exposure to prochloraz resulted in a dose-dependent decrease in direct and combined aromatase activities from control levels with significant reductions being observed in cells exposed to $\geq 0.3 \mu\text{M}$ prochloraz (Fig. 2 B). The response in the indirect aromatase activity experiment was not monotonic in that activity increased in dose-dependent manner up to $0.3 \mu\text{M}$ and then decreased at greater concentrations. At $0.3 \mu\text{M}$, aromatase activity was approximately 4-fold greater than that in the controls but was significantly less in cells treated with $10 \mu\text{M}$ prochloraz. Furthermore, exposure to prochloraz resulted in a dose-dependent decrease in the production of both

E2 and T at all tested concentrations (Fig. 2 B). A statistically significant, positive correlation was observed between E2 and T ($r=0.0.949$; $p=0.003$) but no statistically significant correlation was observed between either of the hormones and indirect aromatase activity.

3.2.3 Aminoglutethimide

Exposure to aminoglutethimide resulted in significantly lesser aromatase activity in both the direct and combined aromatase assays at concentrations $\geq 1 \mu\text{M}$, and maximum decreases in activity were 9.6- and 3.5-fold for the direct and combined assays, respectively (Fig. 3 A). In contrast, activity in the indirect aromatase assay increased up to 9-fold in a dose-dependent manner after exposure to aminoglutethimide. This increase was significant at exposure concentrations $\geq 0.3 \mu\text{M}$ ($p<0.05$). Aminoglutethimide significantly reduced production of both E2 and T relative to controls (Fig. 3 A).

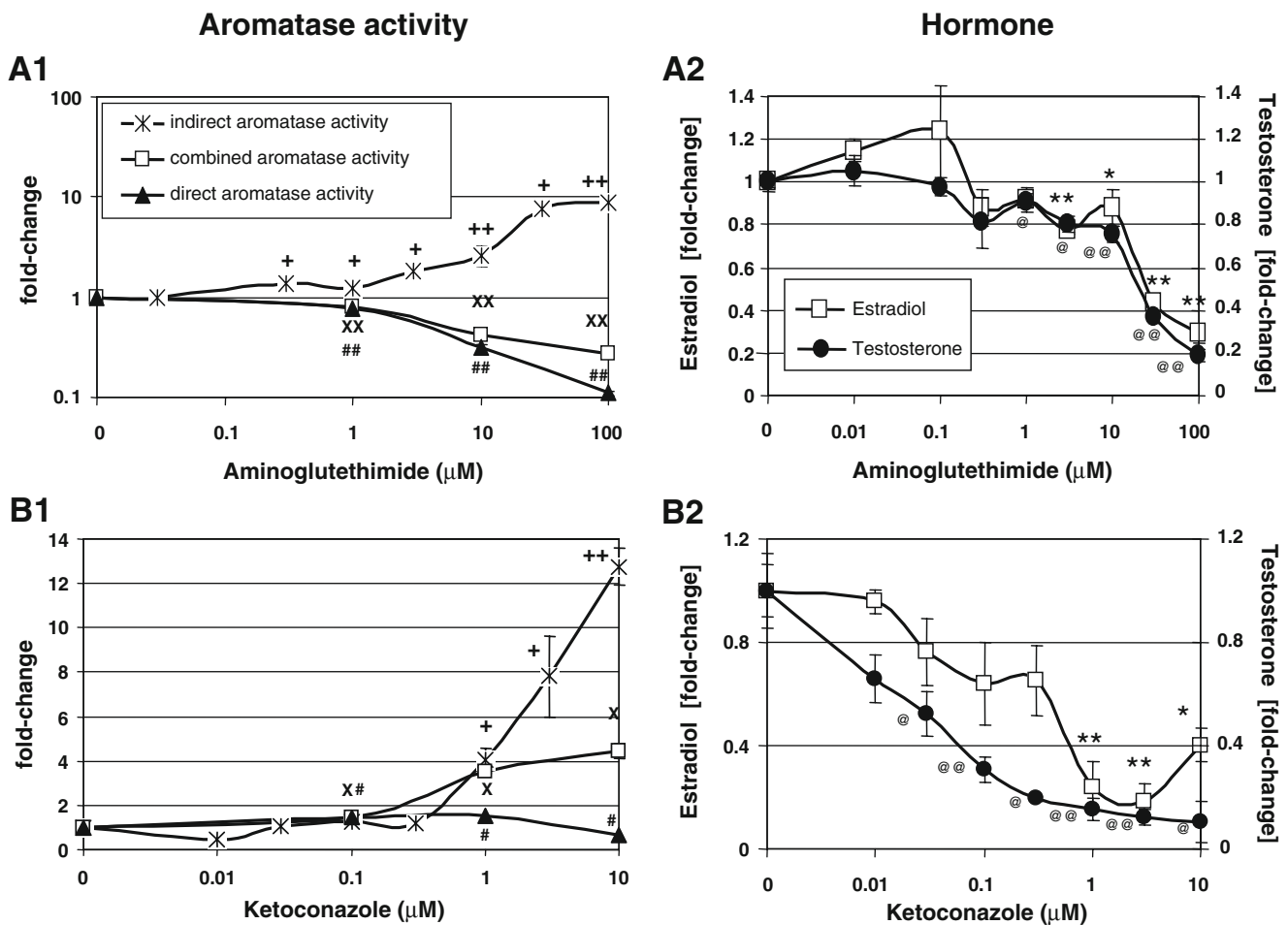


Fig. 3 Weak aromatase inhibitors: effect of ketoconazole and aminoglutethimide on testosterone, estradiol, and direct, combined, and indirect aromatase activity by H295R cells. Cells were treated for 48 h with the indicated concentrations of ketoconazole, aminoglutethimide. Hormone data are expressed as fold changes compared to solvent controls (SC=1). Values represent the mean \pm SEM. Significant

differences for estradiol (asterisks), testosterone (at symbols), and indirect (plus signs), combined (multiplication signs), and indirect (number signs) aromatase activity are reported relative to the solvent control. Multiple symbols indicate different significant levels: one symbol = $p<0.05$; two symbols = $p<0.01$; three symbols = $p<0.001$

Testosterone levels were significantly reduced from controls when exposed to $\geq 1.0 \mu\text{M}$ aminoglutethimide. While aminoglutethimide affected E2 in a manner similar to T at the greatest concentration tested, a 3-fold greater dose was required ($3 \mu\text{M}$) to elicit a significant reduction from control levels. A statistically significant, positive correlation was observed between E2 and T ($r=0.965$; $p<0.001$), while both E2 and T changes were negatively correlated with indirect aromatase activity (E2: $r=-0.939$; $p<0.001$; T: $r=-0.956$; $p<0.001$).

3.2.4 Ketoconazole

Exposure to ketoconazole resulted in dose-dependent reductions in E2, T, and direct aromatase endpoints when compared to SC (Fig. 3 B). In the indirect aromatase assay, a dose-dependent increase in activity occurred in cells exposed to $\geq 1 \mu\text{M}$ ketoconazole. The maximum effect was approximately an 13-fold increase relative to the SCs in cells exposed to $10 \mu\text{M}$. In the combined aromatase assay, activity was also greater than in the SCs with statistically significant differences observed at concentrations $\geq 1 \mu\text{M}$ and the maximum effect of 4-fold occurring at an exposure concentration of $10 \mu\text{M}$. In the direct aromatase assay, changes in aromatase activity were bimodal with significantly greater activities (1.5-fold relative to SC) observed at 0.1 and $1 \mu\text{M}$ and lesser activities than those of the SCs being observed at $10 \mu\text{M}$. Furthermore, statistically significant reductions in E2 and T relative to solvent controls were observed at ketoconazole concentrations ≥ 1 and $\geq 0.03 \mu\text{M}$, respectively. No statistically significant correlations were observed between aromatase and concentrations of E2 or T. However, a statistically significant, positive correlation was observed between concentrations of E2 and T ($r=0.875$; $p=0.013$).

3.2.5 Correlation analysis

The correlation between each set of endpoints was analyzed using the Pearson correlation test (Table 1). The strongest correlations were observed between E2 and the combined

aromatase activity ($r=0.66$; $p<0.001$). This relationship was significantly improved when ketoconazole was removed from the data set ($r=0.84$; $p<0.001$). The combined aromatase activity correlated best with the direct and indirect aromatase activity measurements ($r=0.83$; $p<0.001$ and 0.71 ; $p<0.001$, respectively). T on the other hand only correlated well with E2 ($r=0.64$; $p<0.001$).

Atrazine and forskolin were classified as general inducers because of their ability to stimulate production of E2 and T and aromatase activity in the indirect, but not the direct aromatase assay. Prometon was also classified as a general inducer because of the similarity of E2 and indirect aromatase activity profiles to those observed for forskolin and atrazine. Letrozole, prochloraz, aminoglutethimide, and ketoconazole were classified as inhibitors based of their ability to inhibit aromatase activity in the direct aromatase assay and by reducing production of E2 and T. This group of inhibitors was further broken down into two sub-groups, potent and weak inhibitors. Letrozole and prochloraz were grouped together as potent inhibitors because both chemicals caused effects at concentrations less than $0.03 \mu\text{M}$. Aminoglutethimide and ketoconazole were grouped together as weak inhibitors because no effects were observed in any endpoint until greater than $0.1 \mu\text{M}$.

4 Discussion

4.1 General inducers

4.1.1 Forskolin and atrazine

Forskolin is an extract from the plant *Coleus forskohlii* and has been shown to induce hormone- responsive adenylate cyclase and increase intracellular cyclic AMP (Seamon et al. 1981). Some genes involved in steroidogenesis, including the CYP19 gene expressed in H295R cells, have a cAMP response-element where cAMP can bind and up-regulate gene expression (Watanabe and Nakajin 2004). The results of this study agree with this mechanism of action in that exposure to forskolin resulted in increased indirect and

Table 1 Pearson coefficients

Pearson coefficient	Estradiol	Testosterone	Indirect aromatase	Combined aromatase
Testosterone	0.64 (0.61) ***	–	–	–
Indirect aromatase	0.52 (0.67) ***	0.079 (0.28)	–	–
Combined aromatase	0.66 (0.84) ***	0.30 (0.60)**	0.71 (0.72)***	–
Direct aromatase	0.53 (0.65) ***	0.23 (0.45)*	0.32 (0.35)**	0.83 (0.84)***

Analysis of the correlations between estradiol, testosterone, and indirect, combined, and direct aromatase activity when the data of all seven chemicals are combined. Numbers in parenthesis are the Pearson coefficients without ketoconazole included

* $p<0.05$; ** $p<0.01$; *** $p<0.001$

combined aromatase activity, estradiol, and T concentrations. Furthermore, since forskolin did not directly affect aromatase activity, the greater aromatase activity in the 48-h exposure was most likely not due to direct interactions with the aromatase protein but rather through an increase in intracellular cyclic AMP.

Exposure of H295R cells to atrazine resulted in a similar hormone and aromatase activity dose-response profile as forskolin. Atrazine has been shown to increase aromatase activity in H295R cells after a 24-h exposure (Sanderson et al. 2001). Furthermore, experimental evidence has suggested that the increase in aromatase activity was due to an increase in cAMP (Sanderson et al. 2002). The observed results in the present study are consistent with this mechanism of action for atrazine, because atrazine did not directly affect aromatase activity, but after a 48-h exposure, E2, T, and indirect and combined aromatase activity all increased. However, when compared to forskolin, the effect of atrazine has to be classified as weak due to the much greater concentration required to elicit effects.

In conclusion, the hormone and aromatase activity profiles for forskolin and atrazine were comparable and suggest similar mechanism of action even though exposure to forskolin resulted in greater concentrations of E2, T, and greater aromatase activity at much lesser concentrations than atrazine. Both chemicals increased estradiol levels to a much larger extent than T. These results point out the need to measure more than one endpoint when measuring aromatase activity. For example, if we had only measured the effects of the chemicals directly interacting with the aromatase protein, we would have wrongly concluded that forskolin and atrazine do not affect aromatase activity.

4.2 Potent inhibitors

4.2.1 Letrozole and prochloraz

Letrozole is a selective aromatase inhibitor and is used in breast cancer treatments to reduce circulating levels of E2 that promote growth of the cancer. Consistent with its pharmacological mechanism of action, letrozole directly inhibited aromatase activity and resulted in lesser E2 production in H295R cells in our study. Indirect aromatase activity in the cells exhibited a biphasic response, which is possibly due to a positive feedback mechanism caused by the decrease in E2 production with the cells increasing the amount of aromatase enzyme to make more E2. At greater concentrations of letrozole, there was sufficient letrozole to completely block aromatase activity, while lesser concentrations of letrozole would promote the production of the aromatase protein. This type of biphasic response has been shown in other studies. Villeneuve et al. (2006) found that CYP19A

gene expression in fathead minnow ovaries increased after being exposed to fadrozole, an aromatase inhibitor.

Furthermore, E2 concentrations were less than controls starting at 0.001 μ M letrozole, which is what is expected from a potent aromatase inhibitor. Interestingly, we observed no change in T concentrations at lesser letrozole concentrations and a slight decrease in T at the greatest concentration. This is counter to what would be expected for a specific aromatase inhibitor. Several studies with humans and rats demonstrated that letrozole exposure resulted in an increase in serum T levels (Kumru et al. 2007; Loves et al. 2008). The increase in T levels observed in these studies may have been due to letrozole blocking the production of estrogens and estrogen effects on luteinizing hormone. Estrogens can suppress the secretion of luteinizing hormone in the pituitary gland. Therefore, when estrogen formation is blocked by letrozole, luteinizing hormone can subsequently increase. Greater luteinizing hormone can lead to greater T production in vivo (Loves et al. 2008).

One possible reason that an increase in T after the exposure to letrozole in the H295R cells was not observed is that the cells do not have the same signaling pathways and interaction between organs that is seen in in vivo tests. The H295R cells cannot produce luteinizing hormone and also cannot receive the luteinizing hormone signal from the pituitary gland. This interaction between organs signaling pathways is a good example of the limitations of in vitro testing. Often, in vitro testing only evaluates one type of cell or organ, and the interactions with other types of cells are missed including signaling among the hypothalamus–pituitary–gonadal axis. However, the inhibiting effects of letrozole on E2 production would have been correctly predicted based on the in vitro data presented here.

Prochloraz is a widely used fungicide that has been shown to disrupt the action of several enzymes involved in steroidogenesis, including aromatase and those involved in the metabolism of steroid hormones (Vinggaard et al. 2005; Laignelet et al. 1989). In our study, prochloraz directly inhibited aromatase activity and resulted in less E2 production compared to control. As was observed for letrozole, indirect aromatase activity showed a biphasic response when H295R cells were exposed for 48 h. In contrast to letrozole, inhibition of T was the most sensitive response to prochloraz. Existing studies of rats exposed to prochloraz supported our results and found that serum testosterone levels and CYP17 enzyme activity were reduced (Blystone et al. 2007; Laier et al. 2006; Vinggaard et al. 2005). This suggests that the lesser production of T observed in the H295R cells was caused mainly by the reduced CYP17 enzyme activity, and this would account for the large decrease of T.

In conclusion, letrozole and prochloraz resulted in similar enzyme and estradiol profiles. Both chemicals were

potent aromatase inhibitors that demonstrated a biphasic dose response for indirect aromatase activity when exposed for 48 h. Furthermore, the testosterone profiles between letrozole and prochloraz were slightly different. One theory for this is that prochloraz affects multiple enzymes within the steroidogenic pathway, including aromatase and CYP17, whereas letrozole is a highly specific inhibitor of aromatase. However, to date, there are no known studies that investigated the effects of letrozole on other steroidogenic enzymes such as CYP17, and further research would be required to definitely answer this question.

4.3 Other effectors

4.3.1 Ketoconazole

Ketoconazole is a fungicide that is used mostly in pharmaceutical applications including over-the-counter dandruff shampoo. Ketoconazole has been found to decrease both 17,20-lyase and aromatase activity *in vitro* (Weber et al. 1991) but did not affect 3 β -HSD or 17 β -HSD (Ayub and Stitch 1986). In our study, exposure to ketoconazole resulted in a decrease of E2 and directly inhibited aromatase activity, which is consistent with ketoconazole's previously reported mechanism of action as an aromatase inhibitor. When the cells were exposed for 48 h, greater levels of indirect and combined aromatase activity were observed. As with prochloraz and letrozole, it is hypothesized that ketoconazole most likely increases aromatase activity due to feedback mechanisms triggered by E2 concentrations in response to direct interaction with the aromatase protein. Unlike letrozole and prochloraz, aromatase activity after a 48-h exposure to ketoconazole did not exhibit a biphasic response, and greater aromatase activity was observed even at the greatest concentrations of ketoconazole. Ketoconazole has the ability to affect the aromatase protein directly, as shown in this study, but most likely does not have the same binding affinity as letrozole and prochloraz and cannot shut off the aromatase activity as well at greater concentrations. Furthermore, the lesser concentration of T can be explained by effects on enzymes more upstream in steroidogenesis such as 17,20-lyase. A decrease in T levels is consistent with *in vivo* studies in humans that ingested ketoconazole. For instance, in a study where boys were dosed with ketoconazole for 8 years, significant decreases in serum T production were observed (Almeida et al. 2008).

4.3.2 Aminoglutethimide

Aminoglutethimide is a "generation I" aromatase inhibitor that has also been reported to act as a potent inhibitor of P450 side-chain cleavage. Due to its direct inhibition of

P450 side-chain cleavage and the aromatase enzyme, it was used to treat Cushings syndrome (a disease that causes an increase in cortisol) and breast cancer, respectively (Fassnacht et al. 1998; Foster et al. 1983). This mechanism is consistent with the results of our study in the H295R cells, which is characterized by a general decrease in direct and combined aromatase activity, and subsequently E2 production. Also, we observed a decrease in T, which is what was expected due to inhibition of P450 side-chain cleavage. The 10-fold increase in indirect aromatase activity caused by aminoglutethimide is most likely due to feedback mechanisms that were similarly observed for letrozole, prochloraz, and ketoconazole. In the context of screening chemicals to determine their mechanism of action, care has to be taken when interpreting results if indirect aromatase activity was the only endpoint measured, because of the large increases in aromatase activity by direct-acting aromatase-inhibiting chemicals.

4.3.3 Prometon

Prometon is a widely used nonselective triazine herbicide and has been shown to induce E2 *in vitro* (in H295R cells) and testosterone *in vivo* (fathead minnow reproduction test), respectively (Villeneuve et al. 2007; Villeneuve et al. 2006). While the specific mechanism of action of prometon has never been identified, Villeneuve et al. (2006) found that prometon did not affect the estrogen receptor in MVLN cells or androgen receptor-mediated responses of MDA-kb2 cells. In our study, an increase in E2 concentrations was observed when H295R cells were exposed to greater than 3 μ M concentration of prometon but no change in T was observed. Indirect and combined aromatase activity also increased in the 48-h exposure, which would explain the increase in E2. Interestingly, prometon also reacted directly with the aromatase protein by decreasing enzyme activity. These data clearly distinguish prometon from the other chemicals analyzed in this study because no other chemical both directly inhibited aromatase activity and increased E2 production. A potential mode of action for the observed increase in estradiol caused by prometon might be an interaction with estradiol metabolizing enzymes (i.e., sulfotransferase). However, further experiments are needed to confirm the potential effects of prometon on such metabolizing pathways.

4.3.4 Predictability of aromatase activity for E2 and T

Correlations between E2, T, and indirect, combined, and direct aromatase activity revealed that E2 correlated strongest with the combined aromatase activity ($r=0.66$). A correlation between direct aromatase activity and E2 and between indirect aromatase activity was also observed but

not to the same degree ($r=0.53$ and 0.52 , respectively). This was expected because the combined aromatase activity integrates both indirect and direct effects on the aromatase enzyme. For example, after exposure to ketoconazole, a large increase in indirect aromatase activity was observed. In contrast, a slight decrease in direct aromatase activity occurred. The combined aromatase activity measurement in this case was more intermediate, and a smaller increase was observed than in the indirect aromatase activity. In terms of predicting effects on E2 from aromatase activity, the combined aromatase activity was the best predictor of changes in E2 concentrations. For six out of the seven chemicals measured, combined aromatase activity and E2 followed the same pattern (i.e., when E2 increased combined aromatase activity also increased). Indirect aromatase activity was the least accurate predictor of changes to E2, and for four out of the seven chemicals indirect aromatase activity showed different trends than E2. According to the results presented here the most relevant aromatase activity measurement, and in our opinion the best approach for future studies, is the combined aromatase activity measurement for the prediction of E2 production, although the use of all three aromatase activity measurements would be needed to predict a chemical's mode of action on aromatase activity. The combined aromatase activity measurement was better correlated (i.e., greater r value) with E2 and T than any of the other aromatase measurements.

Furthermore, it was observed that the general inducers, forskolin and atrazine, did not directly affect aromatase activity but acted through indirect mechanisms, as oppose to the general inhibitors in which all chemicals acted directly on the aromatase enzyme. Therefore, direct aromatase did not correctly predict what would happen to E2 hormone production in the general inducers, and indirect aromatase activity did not correctly predict what would happen to E2 hormone production in the inhibitors.

5 Conclusion

In conclusion, the results show that measuring only one or two endpoints can be misleading for the determination of the mechanism of action of a chemical on aromatase activity. For example, indirect aromatase activity increased in all chemicals that were direct aromatase inhibitors. Therefore, by measuring only indirect aromatase activity, it would be easy to falsely conclude that the chemical increases aromatase activity even though one might be measuring a feedback mechanism offsetting direct effects on the enzyme's activity. Additionally, to determine a chemical's mechanism of action directly interacting on the aromatase enzyme, it would be more advantageous to measure the effect of a chemical on direct aromatase activity than measuring indirect aromatase activity

because this assay does not involve any feedback loops. For example, the microsomal aromatase assay that is currently required as part of US-EPA's mandatory EDSP is one assay that measures only direct effects of chemicals on aromatase activity but this assay and others like it would miss effects of chemicals that indirectly affect aromatase activity. Assays like the H295R aromatase assay can adequately address both the direct effects of chemicals and the indirect effects if the recommendations of this manuscript are followed.

In the case of letrozole, comparison of the H295R in vitro assay to in vivo work in humans and rats demonstrated the limitations of in vitro testing in predicting effects in whole animals. Even though the H295R cells exhibit all steroidogenic enzymes, other signals that are not expressed in this cell line (i. e., luteinizing hormone) can make predicting effects in whole animals difficult. Nonetheless, for most of the chemicals, E2, T, and combined aromatase activity were the best predictors of the mechanism of action of the chemical. The only chemical for which combined aromatase activity did not correctly predict effects on E2 production was ketoconazole. In this case, E2, T, and direct aromatase activity were the best predictors of effect. Based on the findings of this study, it is recommended to include all endpoints measured, namely direct, indirect, and combined aromatase activity and E2 and T to be able to correctly predict the mechanism of action for all chemicals.

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