



## A Yeast Screen System for Aromatase Inhibitors and Ligands for Androgen Receptor: Yeast Cells Transformed with Aromatase and Androgen Receptor

Paul Mak,<sup>1</sup> Fernando Dela Cruz,<sup>2</sup> and Shiuan Chen<sup>3</sup>

<sup>1</sup>Wyeth-Ayerst Research, Pearl River, New York, USA; <sup>2</sup>American Cyanamid Company, Princeton, New Jersey, USA; <sup>3</sup>Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, California, USA

Endocrine disruptors are hormone mimics that modify hormonal action in humans and animals. It is thought that some endocrine disruptors modify estrogen and androgen action in humans and animals by suppressing aromatase activity. Aromatase cytochrome P450 is the key enzyme that converts C19 androgens to aromatic C18 estrogenic steroids. We have developed a novel aromatase inhibitor screening method that allows us to identify antiaromatase activity of various environmental chemicals. The screen was developed by coexpressing the human aromatase and the mouse androgen receptor in yeast cells, which carry the androgen-responsive  $\beta$ -galactosidase reporter plasmid. Functional expression of aromatase in yeast has been demonstrated using the [<sup>3</sup>H]-water release assay with intact cells as well as with yeast microsomes. The aromatase activity could be blocked by known aromatase inhibitors such as aminoglutethimide (AG). Yeast-produced androgen receptors were able to transactivate a yeast basal promoter linked to an androgen-responsive element in response to androgens. The resultant triple yeast transformant responded to the treatment of testosterone, androstenedione, or 5 $\alpha$ -dihydrotestosterone (5 $\alpha$ -DHT). In the absence of the aromatase inhibitor AG, transcriptional activation was observed only for the nonaromatizable androgen 5 $\alpha$ -DHT. However, the two aromatizable androgens (testosterone and androstenedione) induced the reporter activity in the presence of AG. Using this yeast-based assay, we confirmed that two flavones, chrysin and  $\alpha$ -naphthoflavone, are inhibitors of aromatase. Thus, this yeast system allows us to develop a high-throughput screening method, without using radioactive substrate, to identify aromatase inhibitors as well as new ligands (nonaromatizable androgen mimics) for the androgen receptors. In addition, this screening method also allows us to distinguish nonandrogenic aromatase inhibitors from inhibitors with androgenic activity. This yeast screening method will be useful to screen environmental chemicals for their antiaromatase activity and for their interaction with androgen receptor. **Key words:** androgen receptor, aromatase, cytochrome P450, endocrine disruptors, yeast screening method. *Environ Health Perspect* 107:855–860 (1999). [Online 28 September 1999]

<http://ehpnet1.niehs.nih.gov/docs/1999/107p855-860mak/abstract.html>

The conversion of androgens to estrogens is catalyzed by the enzyme aromatase, which belongs to the cytochrome P450 superfamily. Aromatase plays an important role in the expression of secondary sexual characteristics, maintenance of pregnancy, and brain sex differentiation (1). Furthermore, the enzyme has received considerable attention in breast cancer development because high expression of the enzyme activity has been associated with a significant number of breast tumors (2–4). Thus, aromatase inhibitors are used as therapeutic agents for the treatment of estrogen-dependent breast cancer by inhibition of estrogen production in addition to antiestrogen treatment.

Aromatase inhibitors can be separated into two classes based on their mechanisms of action. Aromatase inhibitors such as 4-hydroxyandrostenedione (4-OHA) and 1,4,6-androstatrien-3,17-dione (ATD) are steroidal substrate analogs, which inhibit the enzyme

activity by competing for the active site of the enzyme (5). The other class of inhibitors is nonsteroidal inhibitors such as aminoglutethimide (AG), which interact with the heme prosthetic group of aromatase (6).

Endocrine disruptors are hormone mimics that modify hormonal action in humans and animals (7–9). These compounds include chemicals isolated from plants (such as phytoestrogens) and man-made chemicals (such as dioxins and polychlorinated biphenyls). There is increasing concern about how many such chemicals are present in our environment. There is a need for high throughput approaches to examine various environmental chemicals. Endocrine disruptors bind to the estrogen receptor or androgen receptor and induce many components of estrogen or androgen action (10–12). We feel that it is also possible that some of these compounds may act in an indirect fashion by inhibiting aromatase activity, resulting in a decrease in

the level of estrogens or an increase in the level of androgens in cells. Research from other laboratories as well as our own has demonstrated that phytoestrogens such as flavones are competitive inhibitors of aromatase (13–15). Some flavones can inhibit aromatase with  $K_i$  values similar to that of AG, an approved drug for treating breast cancer. It is possible that there are additional environmental chemicals that can suppress aromatase activity by competing for the binding of the androgen substrates or by interacting with the heme group of aromatase.

The conventional method for aromatase inhibitor screening uses an *in vitro* enzyme assay with human placental microsomes as sources of the enzyme. However, the aromatase assay in such preparations depends on several components including short incubation time, the stability of NADPH-cytochrome P450 reductase and aromatase in such preparations. Furthermore, the assay procedure is tedious and it would not be easy to adapt this assay for high-throughput screening. Although certain intact cells expressing aromatase, including human aromatase transfected mammalian cell lines (16), provide an ideal *in vivo* model for aromatase inhibitor screening, the cost of mammalian cell culture is a concern. In addition, these expression systems require an enzyme assay that uses radioactive substrates, which is a limiting factor for high-throughput screening. To circumvent these problems, we developed a novel method, which does not involve a radioactive assay, by coexpressing the human aromatase enzyme and the mouse androgen receptor in yeast cells carrying the androgen-responsive reporter plasmid. We also provide evidence that this high-throughput screening method can differentiate aromatase inhibitors with or without androgenic activity.

Address correspondence to S. Chen, Division of Immunology, Beckman Research Institute of the City of Hope, 1450 E. Duarte Road, Duarte, CA 91910 USA. Telephone: (626) 359-8111, ext. 2601. Fax: (626) 301-8186. E-mail: schen@smtplink.coh.org

S.C. was supported by NIEHS grant ES08258.

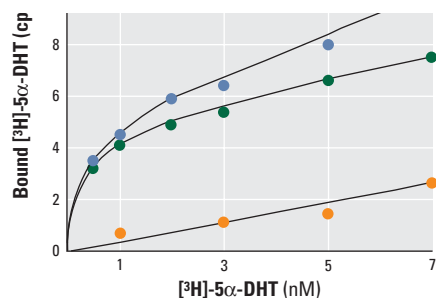
Received 26 February 1999; accepted 17 June 1999.

## Materials and Methods

**Chemicals and buffers.** Restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolab (Beverly, MA). Taq polymerase was from Perkin-Elmer Cetus (Norwalk, CT). 1,2- $^3\text{H}$ -Androstenedione (55 Ci/mmol) and 1,2,5,6,7- $^3\text{H}$ -5 $\alpha$ -dihydrotestosterone (5 $\alpha$ -DHT) (120 Ci/mmol) were from DuPont-New England Nuclear (Boston, MA). AG, 4-OHA, and *o*-nitrophenyl- $\beta$ -D-galactopyranoside were obtained from Sigma (St. Louis, MO). Testosterone, androstenedione, 1,4,6-androstatrien-3,17-dione and other steroids were obtained from Steraloids (Newport, RI), yeast nitrogen base and dextrose from Difco (Detroit, MI), Glassperlen from B/Braun Melsungen AG (Melsungen, Germany), and 5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactoside (x-gal) from Bio-Rad (Hercules, CA).

**Transcription buffer for  $\beta$ -galactosidase assay.** We used sodium phosphate (0.12 M dibasic), 0.04 M sodium phosphate (monobasic), 10 mM potassium chloride, 1 M magnesium sulfate, and 0.27% 2-mercaptoethanol, pH 7.0 (room temperature), for the transcription buffer.

**Yeast strains.** We used the *Saccharomyces cerevisiae* strain BJ2168 (Mat a, a *prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52*) (17).



**Figure 1.** (A) Androgen receptor expression plasmid (YEPArc). Abbreviations: CUP1, copper-inducible promoter; CYC1, iso-1-cytochrome *c* gene; GRE/PRE, glucocorticoid/progesterone response element; LEU2, leucine selectable marker; TDH3, triose phosphate dehydrogenase; TRP1, tryptophan selectable marker; UBI, ubiquitin; URA3, uracil selectable marker. The mouse androgen receptor cDNA was inserted into the yeast expression plasmid to produce a UBI-fusion receptor under the control of the CUP1. (B) Yeast reporter plasmid (YEPA). The yeast upstream regulatory elements of the CYC1 promoter were replaced by an oligonucleotide containing two copies of an androgen-responsive element (GRE/PRE) inserted into the *Xho*I site upstream of the CYC1 promoter, which is fused to the *lac-Z* gene of *Escherichia coli*. (C) Aromatase expression plasmid (YEPAro). The human aromatase cDNA was inserted into the yeast expression vector to produce an UBI-fusion protein under the control of the TDH3 promoter. All three vectors are 2-micron plasmids.

Expression of the human aromatase was under the control of a constitutive yeast promoter, triosephosphate dehydrogenase (TDH3); expression of the mouse androgen receptor was under the control of a regulated yeast promoter, metallothionein promoter (CUP1) as described previously (18). Growth and transformation of yeast cells were performed according to standard procedures (19).

**Construction of yeast expression vector for the mouse androgen receptor.** A cassette that contained the metallothionein promoter (CUP1)-ubiquitin gene-linker containing unique *Eag*I and *Bss*HIII sites followed by the iso-1-cytochrome C (CYC1) terminator was inserted into the *Bam*HI and the *Sph*I sites (multiple cloning sites) of the yeast vector (YEPA351). The mouse androgen receptor cDNA was excised from the previously described yeast expression vector (YEPAmar) (20) after complete digestions with *Eag*I and *Bss*HIII and was subsequently ligated to YEPA351. The resultant androgen receptor expression vector (YEPArc) (Figure 1A) was used to transform the yeast strain, BJ2168, using lithium acetate protocol (19). Transformants were selected by leucine auxotrophy.

**Construction of androgen-responsive reporter plasmid.** The reporter plasmid (YEPA) was used to study the transcriptional activation of the mouse androgen receptors produced in yeast cells (20). Briefly, an oligonucleotide containing two copies of the glucocorticoid/progesterone response element (GRE/PRE) from the tyrosine amino-transferase gene (21) was inserted into the unique *Xho*I site of the yeast reporter plasmid, pC1, as described previously (18,20). The resulting reporter plasmid (YEPA) (Figure 1B) contains an androgen response element (GRE/PRE) upstream of the CYC1 promoter that is fused to the *lac-Z* gene of *Escherichia coli*. These plasmids were used to transform yeast cells expressing the mouse androgen receptor and the final transformants (YEPArc/YEPA) were selected by leucine and uracil auxotrophy.

**Construction of yeast expression vector for human aromatase.** Two unique restriction sites were engineered at the 5' and the 3' ends of the human placental aromatase coding sequence (16,22) for insertion into the yeast expression vector (YEPAV5) described previously (18). This was achieved by PCR-mediated mutagenesis.

The two primers used to amplify the aromatase gene (1.5 kb) were 5'-ACTTCACGGCCGATGGT TTTTGAAATGCTGAACCA-3' (forward) and 5'-ACTTCACACACAGTGCTAGTGTCCAGACACCTGTC-3' (reverse).

The resultant aromatase cDNA contained an *Eag*I site in front of the start codon

and a *Dra*III site right after the stop codon. To produce an in-frame ubiquitin-fusion receptor under the control of the yeast TDG3 promoter, an oligonucleotide containing the last six amino acids of the ubiquitin gene followed by an *Eag*I and a *Dra*III site was inserted into the AfIII-Kpn sites of the yeast expression vector, YEPAV5. After complete digestions with *Eag*I and *Dra*III, the aromatase cDNA was inserted into the yeast expression vector (YEPAro) (Figure 1C) was used to transform BJ2168 yeast strain carrying the androgen receptor expression construct and the androgen-responsive reporter plasmid (YEPArc/YEPA). The final triple transformant yeast strain (YEPArc/YEPAro/YEPA) was selected by leucine, uracil, and tryptophan auxotrophy.

**Aromatase assay.** The aromatase activity was measured by [ $^3\text{H}$ ]-water assay (23) directly on yeast cultures. The [ $^3\text{H}$ ]-water release assay for human aromatase expressed in mammalian cells and yeast was validated in our laboratory by a product isolation assay (16,22). [ $^3\text{H}$ ]-Androstenedione at a final concentration of 46 nM was added to 1 mL transformed yeast strain (YEPArc/YEPAro/YEPA) when cell density reached the late log phase. After 1-hr incubation at 30°C, the reaction was stopped by addition of 200  $\mu\text{L}$  10% (weight/volume) trichloroacetic acid and 100  $\mu\text{L}$  dextran-coated charcoal (2% norit A and 0.2% dextran in water). After 10 min incubation, the suspension was centrifuged and the supernatant was again treated with dextran-coated charcoal. After centrifugation, the supernatant was filtered through a small column fitted with glass wool and the tritiated water content was determined by scintillation counting. For the *in vitro* enzyme assay, microsomes were prepared from yeast cells as follows: Yeast cells were washed with enzyme buffer (50 mM Tris-HCl and 1 mM EDTA, pH 7.0) and disrupted with glass beads. This and subsequent procedures were performed at 4°C. The cell suspension was centrifuged at 1,000  $\times g$  for 10 min, and the supernatant was centrifuged at 10,000  $\times g$  for 30 min. The resulting supernatant was further centrifuged at 100,000  $\times g$  for 30 min to obtain the yeast microsomal pellet. Yeast microsomes (20  $\mu\text{L}$ ) were added to 180  $\mu\text{L}$  enzyme buffer containing NADPH (0.2 mg/mL) and [ $^3\text{H}$ ]-androstenedione at concentration as indicated. After 20 min incubation at 37°C, the reaction was stopped by the addition of 20  $\mu\text{L}$  50% trichloroacetic acid. The rest of the procedure was identical to that described for the yeast culture assay.

**Preparation of yeast extracts and receptor-binding assays.** Yeast extracts were prepared for androgen receptor-binding assay as described previously (18,20).

**Transcription assays.** The triple transformant yeast strain was grown in enriched medium (without tryptophan, uracil, and leucine) containing 20 nM androstenedione in the presence or absence of aromatase inhibitors. When cell density reached the late log phase, cells were harvested by centrifugation and subsequently disrupted by vortexing with glass beads. Cell suspension was centrifuged at  $10,000 \times g$  for 10 min and the supernatant (yeast extract) was assayed for  $\beta$ -galactosidase activity as described previously (18,20).

**Plate assay for high-throughput screening.** An overnight culture yeast strain (YEpmARc/YEpARO/YEpA2) (5%) was inoculated into dextrose-agar medium maintained at 50°C. After the addition of vitamins, amino acids, x-gal, and 20 nM androstenedione, the final culture was poured onto a 15-cm petri plate and allowed to cool at room temperature. Discs containing different aromatase inhibitors were placed on top of the agar and the plate was incubated for 2 days at 30°C. As a control, another culture plate containing the same constituents without 20 nM androstenedione was also set up simultaneously to detect the androgenic activity of aromatase inhibitors.

## Results and Discussion

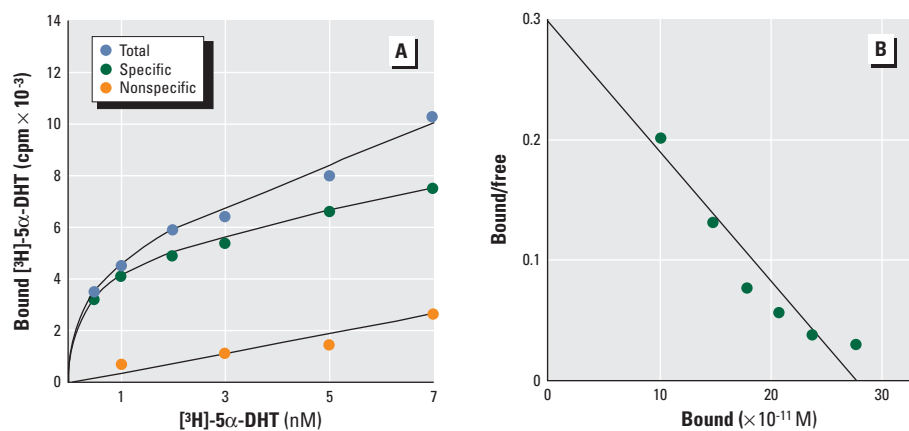
**Concept of coexpressing androgen receptor and aromatase in yeast as a novel screening method for antiaromatase activity.** In the present study, we developed a screening method to identify aromatase inhibitors without using an enzyme assay with radioactive substrates. This concept was achieved by coexpressing androgen receptor and aromatase in yeast cells carrying the androgen-responsive reporter plasmid. If the triple transformant yeast cells carrying the three plasmids as described in Figure 1 are incubated with an aromatizable

androgen (testosterone or androstenedione), the androgen diffuses into the cell, where it will either bind to the yeast-produced androgen receptor or will be converted to estrogen by aromatase produced in yeast. If the conditions (concentrations and choice of substrates) favor the enzymatic reactions, most of the androgen will be converted into estrogen but not bind to the androgen receptor and therefore cannot transactivate the yeast basal promoter linked to the androgen-responsive element (GRE/PRE). The end result of this androgen metabolism within the yeast cells is the inhibition or reduction of reporter enzyme  $\beta$ -galactosidase induction. However, androgen-dependent transcriptional activation will be apparent as reflected by the reporter enzyme induction if an aromatase inhibitor is included in the yeast medium. As a first step toward the development of this novel screen, the two expression systems for androgen receptor and aromatase in yeast must be characterized and validated separately.

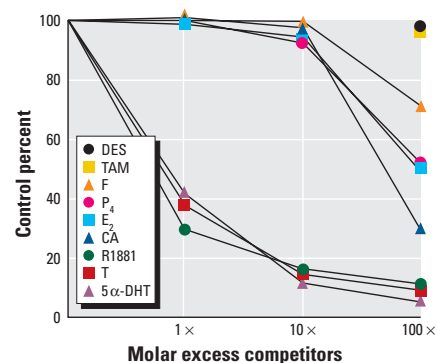
**Androgen-dependent transcriptional activation in yeast.** We have previously shown that many of the steroid/retinoid receptor superfamily can function as ligand-activated transcription factors in yeast cells (18,20,24,25). In this study, a new yeast vector carrying the CUP1 promoter and a *LEU2* gene was constructed to express the mouse androgen receptor. To confirm receptor expression, yeast extracts were prepared from the yeast strain carrying this expression vector (YEpmARc) under copper induction and analyzed for receptor-binding activities. Scatchard analysis indicated that the yeast-produced mouse androgen receptor binds to androgen with high affinity with a  $K_d$  value of 0.96 nM and a maximum binding capacity of 135 fmol/mg (Figure 2). The binding

specificity of these receptors was also examined by labeling the extracts with 10 nM [ $^3$ H]-5 $\alpha$ -DHT in the presence or absence of 1-, 10- and 100-fold molar excess of radioinert competitors (Figure 3). Yeast-produced androgen receptors exhibit steroid-binding specificity characteristics of the authentic mammalian androgen receptor. The functional expression of the androgen receptor in yeast was further confirmed by transforming the yeast strain (YEpmARc) with a reporter plasmid containing two copies of a GRE/PRE of the glucocorticoid responsive tyrosine aminotransferase gene upstream of the yeast promoter, *CYC1*, which is fused to the *lac-Z* gene of *E. coli*. The resulting double transformant yeast strain (YEpmARc/YEpA) was analyzed for ligand-dependent transcriptional activation. In the presence of 10  $\mu$ M cupric sulfate, the three androgens (androstenedione, testosterone, or 5 $\alpha$ -DHT) were able to induce the  $\beta$ -galactosidase activity in the absence or presence of AG (1 mM) (Figure 4). These results indicate that aromatase inhibitors such as AG do not interfere with the binding of androgens to the expressed androgen receptor. We also observed that the reporter enzyme could be induced (3- to 4-fold) by high concentrations of androgen ( $\geq 1 \mu$ M) in yeast cells in the absence of copper, indicating that the CUP1 promoter is slightly leaky (data not shown).

**Expression of aromatase in yeast.** Baker's yeast (*S. cerevisiae*) is an ideal host for expressing mammalian cytochrome P450s, including aromatase (22). In the present study, we used the previously described ubiquitin-fusion technology (18,20,21) to



**Figure 2.** Saturation analysis of yeast-produced mouse androgen receptor. Abbreviations: 5 $\alpha$ -DHT, 5 $\alpha$ -dihydrotestosterone; NS, nonspecific binding; S, specific binding; T, total binding. (A) Yeast extracts prepared from a yeast strain carrying the androgen receptor expression plasmid (YEpmARc) under induced condition (+ copper) were incubated with increasing concentrations of [ $^3$ H]-5 $\alpha$ -dihydrotestosterone in the absence (T) or presence (NS) of a 100-fold molar excess of radioinert 5 $\alpha$ -DHT. S was determined by NS - T. (B) Scatchard plot analysis of the data from (A). Two experiments gave similar apparent  $K_d$  values.  $K_d = 9.6 \times 10^{-10}$  M.



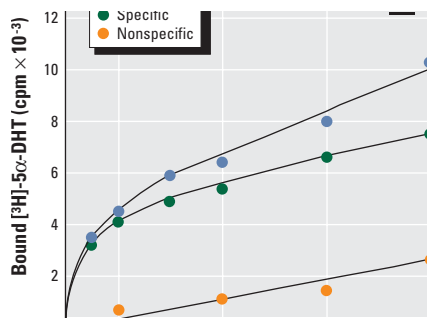
**Figure 3.** Steroid binding specificity of yeast-produced mouse androgen receptor. Abbreviations: 5 $\alpha$ -DHT, 5 $\alpha$ -dihydrotestosterone; DES, diethylstilbestrol; T, testosterone; R1881, methyltrienolone; CA, cyproterone acetate; E<sub>2</sub>, 17 $\beta$ -estradiol; P<sub>4</sub>, progesterone; F, flutamide; TAM, tamoxifen. Aliquots of yeast extracts containing the recombinant mouse androgen receptor were incubated with 10 nM [ $^3$ H]-5 $\alpha$ -DHT in the absence (100% control) or presence of a 1-, 10-, or 100-fold molar excess of radioinert 5 $\alpha$ -DHT, T, R1881, CA, E<sub>2</sub>, P<sub>4</sub>, F, TAM, or DES. Bound [ $^3$ H]-5 $\alpha$ -DHT was determined as described in "Materials and Methods." Values represent the average of two experiments.

express human aromatase functionally in yeast cells. Because a unique *EagI* restriction site was created in front of the start codon of the aromatase gene during the construction of the ubiquitin-fusion protein, the resulting recombinant aromatase contains two extra amino acids (arginine and proline) in front of the methionine after the ubiquitin (76 amino acids) has been cleaved off by the host enzyme. To test whether this recombinant aromatase produced by yeast is functional, aromatase enzyme activity was determined in yeast microsomes by the [<sup>3</sup>H]-water release assay. A double-reciprocal plot of the enzyme activity revealed an apparent Michaelis-Menton constant ( $K_m$ ) of 50 nM and a maximum velocity of 6 pmol [<sup>3</sup>H]-water release/min/mg microsomal protein (Figure 5). The  $K_m$  value is similar to those reported for aromatase in human placental microsomes, but the  $V_{max}$  is lower than those reported previously in placental microsomes (26). Paradoxically, this lower level of enzyme expression in yeast actually enhances the sensitivity of the screen because reporter enzyme induction can be detected in the presence of lower concentrations of aromatase inhibitor.

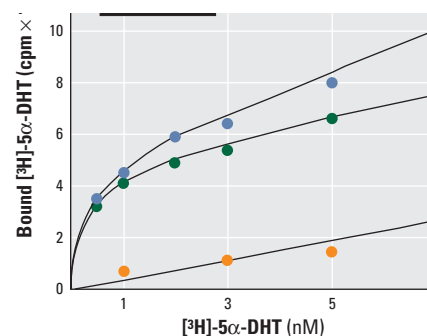
The three aromatase inhibitors (4-OHA, ATD, and AG) were tested on the yeast strain carrying the aromatase expression construct (YEPAO). As shown in Figure 6, 4-OHA and ATD were effective inhibitors, with a median inhibitory concentration of 0.1  $\mu$ M. These two inhibitors were able to inhibit 90% of the aromatase enzyme activity at a concentration of 1  $\mu$ M, whereas the nonsteroidal inhibitor AG required 1 mM to achieve a similar inhibitory effect. Relatively high concentrations of aromatase inhibitors were needed to suppress aromatase in yeast. This observation is likely because of the poor ability of the compounds to diffuse into the yeast cells. We are evaluating approaches to increase the sensitivity of the assay. Although aromatase inhibitor screening using the yeast strain expressing the human aromatase would be a good approach because of rapid cell growth and the stability of the expression plasmid attributed by the auxotrophic marker, this screening method involves an enzyme assay, which will require a large quantity of radioactive substrates when high-volume screening is desired. An ideal aromatase inhibitor screening should handle a large number of samples with good selectivity, sensitivity, and capacity. To accomplish this goal, we coexpressed the androgen receptor and the aromatase enzyme in yeast cells carrying the androgen-responsive reporter plasmid.

**Coexpressing aromatase and androgen receptor in yeast.** The yeast strain expressing the aromatase enzyme was cotransformed with the androgen receptor expression vector

and the androgen-responsive reporter plasmid. The three androgens (testosterone, androstenedione, and 5 $\alpha$ -dihydrotestosterone) were tested for reporter enzyme induction in the triple transformant yeast strain (YEPArc/YEPAro/YEPA). As shown in Figure 7, the aromatizable androgens androstenedione and testosterone were not able to induce the reporter enzyme in the absence of the aromatase inhibitor, AG. However, ligand-dependent transcriptional activation was apparent in the presence of AG (1 mM). Addition of AG alone in the yeast culture did not induce reporter enzyme activity. In contrast to aromatizable androgens, the nonaromatizable androgen, 5 $\alpha$ -DHT, induced the reporter gene efficiently in the presence or absence of AG. Thus, these data clearly support our concept of coexpressing the androgen receptor and the



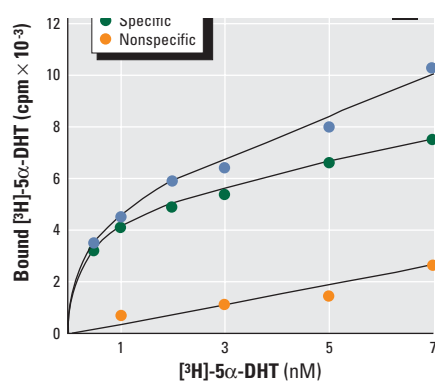
**Figure 4.** Androgen-dependent transcriptional activation in yeast. Abbreviations: Ad, androstenedione; AG, aminoglutethimide; C, control; DHT, 5 $\alpha$ -dihydrotestosterone; T, testosterone. The three androgens (Ad, T, and DHT) were incubated with the yeast strain carrying the mouse androgen receptor expression plasmid and the reporter plasmid (YEPArc/YEPA) under induced conditions in the absence or presence of the aromatase inhibitor, AG (1 mM). Reporter enzyme ( $\beta$ -galactosidase) activities were measured in yeast extracts. Values represent the average of two to three experiments. Yeast culture without androgen incubation was used as the control.



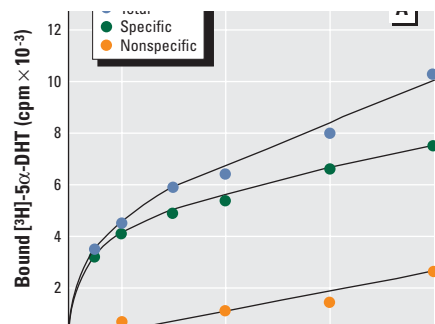
**Figure 5.** Enzyme kinetic analysis of yeast-produced aromatase.  $K_m = 50$  nM.  $V_{max} = 6$  pmol [<sup>3</sup>H]-H<sub>2</sub>O/min/mg protein. The aromatase enzyme activity was determined in microsomes prepared from the yeast strain carrying the aromatase expression plasmid (YEPAO) using the tritiated water assay described in "Materials and Methods."

aromatase enzyme in yeast as a novel aromatase inhibitor screen.

To develop a high-throughput screening assay, the yeast strain carrying the three plasmids (YEPArc, YEPAro, and YEPA) were inoculated on agar plates, which contained the chromogenic substrate, x-gal, and androstenedione (20 nM). Discs containing 10  $\mu$ g of the three aromatase inhibitors, 4-OHA, ATD, and AG, were placed on top of the agar (Figure 8A). After 48 hr incubation at 30°C, blue halo formations as a result of  $\beta$ -galactosidase induction were detected surrounding these inhibitors. To distinguish androgenic from nonandrogenic aromatase inhibitors, the three inhibitors were also tested with the same yeast strain in the absence of androstenedione (Figure 8B). Both 4-OHA and ATD exhibited transcriptional activation as reflected by the formation of



**Figure 6.** Inhibition of aromatase activity in yeast cells. Abbreviations: AG, aminoglutethimide; ATD, 1,4,6-androstatrien-3,17-dione; 4-OHA, 4-hydroxyandrostenedione. Yeast cells carrying the aromatase expression plasmid (YEPAO) were incubated with the substrate, [<sup>3</sup>H]-androstenedione (46 nM) in the absence or presence of an increasing concentration of the aromatase inhibitors, 4-OHA, ATD, and AG. Assay conditions were described in "Materials and Methods." Enzyme activity in the absence of inhibitors was taken as 100%.



**Figure 7.** The three androgens (Ad, T, and DHT) were tested in the triple transformant yeast strain (YEPArc/YEPAro/YEPA) under induced conditions in the absence or presence of the aromatase inhibitor, AG (1 mM), as described in Figure 5. Abbreviations: Ad, androstenedione; AG, aminoglutethimide; C, control; 5 $\alpha$ -DHT, 5 $\alpha$ -dihydrotestosterone; T, testosterone. Values represent the average of two to three experiments.

blue halos surrounding the discs, whereas the nonsteroidal inhibitor, AG, was inactive on this control plate. The observation indicates that 4-OHA and ATD are androgen mimics. The detection of an androgenic activity of these inhibitors may be due to the fact that a rather high amount of compounds was used. These compounds reportedly have weak androgenic activity (27–30). The binding affinity of 4-OHA to the androgen receptor is 0.1% that of mibolerone (30). Using this yeast screening method, we confirmed our previous report (13) that two endocrine disruptors, chrysin and  $\alpha$ -naphthoflavone, are aromatase

inhibitors (Figure 9). Naringenin was weakly positive in the yeast screening method. We will evaluate the method using other compounds that inhibit aromatase. For a high-throughput screening, compounds from a 96-well plate can be delivered by a robot onto a large agar plate that can hold 576 samples. Thus, a large number of compounds can be screened each day.

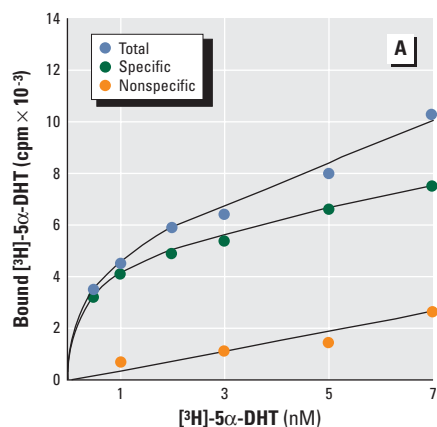
The sensitivity of this screening assay also depends on the choice and concentrations of substrates used because residual substrates will lead to high background. We found that androstenedione is a better substrate than testosterone and that the optimal concentration of androstenedione used is 20 nM. In theory, the androgen receptor and the androgen response element can be substituted with the estrogen receptor and the estrogen response element for this nonradioactive screen. In the latter case, an aromatizable androgen is converted to estrogen, which binds to the estrogen receptor and subsequently induces the reporter enzyme. On a plate assay, the yeast cells will turn blue as a result of reporter enzyme induction. The clear halo will appear in the presence of an aromatase inhibitor. In practice, it is more difficult to identify activity against a blue background. Furthermore, the screen using estrogen receptor expression does not provide information on androgenicity of the inhibitor. In addition, it seems that there are more estrogenic environmental chemicals identified than androgenic chemicals. This will create problems in such yeast screening in that aromatase noninhibitors cannot be distinguished from estrogen mimics. Thus,

we have chosen the androgen-responsive transcription unit for this screen development.

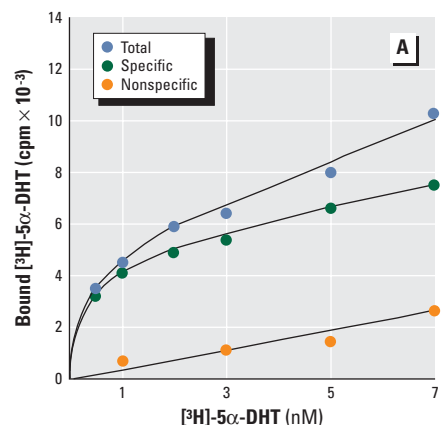
In summary, we have reconstituted for the first time a yeast transcription unit to study androgen action and aromatase simultaneously. This reconstituted system allows us to develop a high-throughput screening method without using radioactive substrate to identify aromatase inhibitors as well as new ligands (nonaromatizable androgen mimics) for the androgen receptors. In addition, this screening method also allows us to distinguish nonandrogenic aromatase inhibitors from inhibitors with androgenic activity. This yeast screening method will be useful to screen environmental chemicals for their antiaromatase activity and for their interaction with androgen receptor.

## REFERENCES AND NOTES

- MacLusky NJ, Clark AS, Naftolin F, Goldman-Rakic PS. Estrogen formation in the mammalian brain: possible role of aromatase in sexual differentiation of the hippocampus and neocortex. *Steroids* 50:459–474 (1987).
- Miller WR, O'Neill J. The importance of local synthesis of estrogen within the breast. *Steroids* 50:537–548 (1987).
- Silva MC, Rowlands MG, Dowsett M, Gusterson B, McKinna JA, Fryatt I, Coombes RC. Intratumoral aromatase as a prognostic factor in human breast carcinoma. *Cancer Res* 49:2588–2591 (1989).
- Reed MJ, Owen AM, Lai LC, Coldham NG, Ghilchik MW, Shaikh NA, James VHT. In situ oestrone synthesis in normal breast and breast tumor tissues: effect of treatment with 4-hydroxyandrostenedione. *Int J Cancer* 44:233–237 (1989).
- Brodie AMH, Schwarzel WC, Shaikh AA, Brodie H. The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen dependent processes in reproduction and breast cancer. *Endocrinology* 100:1684–1695 (1977).
- Salhanick HA. Basic studies on aminoglutethimide. *Cancer Res* 42 (suppl):S3315–S3321 (1982).
- De Rosa C, Richter P, Pohl H, Jones DE. Environmental exposures that affect the endocrine system: public health implications. *J Toxicol Environ Health B Crit Rev* 1:3–26 (1998).
- Santti R, Makela S, Strauss L, Korkman J, Kostian ML. Phytoestrogens: potential endocrine disruptors in males. *Toxicol Ind Health* 14:223–237 (1998).
- Toppari J, Skakkebaek NE. Sexual differentiation and environmental endocrine disruptors. *Baillieres Clin Endocrinol Metab* 12:143–156 (1998).
- Soto AM, Michaelson CL, Prechtl NV, Weill BC, Sonnenschein C, Olea-Serrano F, Olea N. Assays to measure estrogen and androgen agonists and antagonists. *Adv Exp Med Biol* 444:9–23 (1998).
- Kelce WR, Gray LE, Wilson EM. Antiandrogens as environmental endocrine disruptors. *Reprod Fertil Dev* 10:105–111 (1998).
- Sonnenschein C, Soto AM. An updated review of environmental estrogen and androgen mimics and antagonists. *J Steroid Biochem Mol Biol* 65:143–150 (1998).
- Kao YC, Zhou C, Sherman M, Loughton CA, Chen S. Molecular basis of the inhibition of human aromatase (estrogen synthetase) by flavone and isoflavone phytoestrogens: a site-directed mutagenesis study. *Environ Health Perspect* 106:85–92 (1998).
- Kellis JT, Vickery LE. Inhibition of estrogen synthetase (aromatase) by flavones. *Science* 225:1032–1034 (1984).
- Wang C, Makela T, Hase T, Adlercreutz H, Kurzer MS. Lignans and flavonoids inhibit aromatase enzyme in human preadipocytes. *J Steroid Biochem Mol Biol* 50:205–212 (1994).
- Zhou D, Pompon D, Chen S. Stable expression of human aromatase complementary DNA in mammalian cells: a useful system for aromatase inhibitor screening. *Cancer Res* 50:6949–6954 (1990).
- Moehle CM, Ayanardi MW, Kolodny MR, Park FJ, Jones



**Figure 8.** Coexpression of androgen receptor and aromatase in yeast as a high throughput screen to identify aromatase inhibitors. Abbreviations: AG, aminoglutethimide; ATD, 1,4,6-androstatrien-3,17-dione; 4-OHA, 4-hydroxyandrostenedione. (A) The yeast strain (YEpARc/YEpARO/YEpA) was inoculated in medium containing 5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactoside and 20 nM androstenedione as described in “Materials and Methods.” Discs containing 10  $\mu$ g of the aromatase inhibitors 4-OHA, ATD, or AG were placed on top of the agar plate, which was then incubated for 48 hr at 30°C. Blue halos surrounding the discs indicate  $\beta$ -galactosidase enzyme induction. (B) The three aromatase inhibitors (4-OHA, ATD, and AG) were tested on the same yeast strain under the same conditions as described in (A) except that androstenedione was omitted from the medium.



**Figure 9.** Testing of endocrine disruptors in the yeast screening assay. Abbreviations: AG, aminoglutethimide; C, chrysin; N, naringenin;  $\alpha$ -N, naphthoflavone. Three flavones (C, N, and  $\alpha$ -N) and the aromatase inhibitor (AG) were tested in the assay in the presence of 20 nM androstenedione, under conditions described in Figure 8. The numbers in the brackets indicate micrograms of compounds on the discs. Blue halos surrounding the discs indicate  $\beta$ -galactosidase enzyme induction.

- EW. Protease B of *Saccharomyces cerevisiae*: isolation and regulation of the PRB1 structural gene. *Genetics* 115:255–263 (1987).
18. Mak P, McDonnell DP, Weigel NL, Schrader WT, O'Malley BW. Expression of functional chicken progesterone receptor in yeast (*Saccharomyces cerevisiae*). *J Biol Chem* 264:21613–21618 (1989).
  19. Sherman F, Fink GR, Hicks JB. *A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1982.
  20. Mak P, Young CYF, Tindall DJ. A novel yeast expression system to study androgen action. *Recent Prog Horm Res* 49:347–452 (1994).
  21. Bagchi MK, Elliston JF, Tsai SY, Edwards DP, Tsai MJ, O'Malley BW. Steroid hormone-dependent interaction of human progesterone receptor with its target enhancer element. *Mol Endocrinol* 2:1221–1229 (1988).
  22. Pompon D, Liu RYK, Besman MJ, Wang PL, Shively JE, Chen S. Expression of human placental aromatase in *Saccharomyces cerevisiae*. *Mol Endocrinol* 3:1477–1487 (1989).
  23. Thompson EA Jr, Siiteri PK. Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione. *J Biol Chem* 249:5364–5372 (1974).
  24. Mak P, Fuernkranz HA, Ge R, Karathanasis SK. RXR $\alpha$  homodimers function as transcriptional activators in yeast. *Gene* 145:129–133 (1994).
  25. Salerno AJ, He Z, Goos-Nilsson A, Ahola H, Mak P. Differential transcriptional regulation of the *apoA1* gene by retinoic acid receptor homo- and heterodimers in yeast. *Nucleic Acid Res* 24:566–572 (1996).
  26. Kao YC, Higashiyama T, Yarborough C, Osawa Y, Chen S. Functional characterization of 102-amino acid-deleted form of human aromatase. *Steroids* 64:422–429 (1999).
  27. Dowsett M. Clinical development of aromatase inhibitors for the treatment of breast and prostate cancer. *J Steroid Biochem Mol Biol* 37:1037–1041 (1990).
  28. Brodie A. Aromatase and its inhibitors—an overview. *J Steroid Biochem Mol Biol* 40:255–261 (1991).
  29. Dowsett M, Coombes RC. Second generation aromatase inhibitor—4-hydroxyandrostenedione. *Breast Cancer Res Treat* 30:81–87 (1994).
  30. Davies JH, Shearer RJ, Rowlands MG, Poon GK, Houghton J, Jarman M, Dowsett M. Effects of 4-hydroxyandrost-4-ene-3,17-dione and its metabolites on 5 alpha-reductase activity and androgen receptor. *J Enzyme Inhib* 6:141–147 (1992).

## Environmental Health Perspectives online at the



<http://ehp.niehs.nih.gov/>