Exemestane’s 17-hydroxylated metabolite exerts biological effects as an androgen

Eric A. Ariazi,1,2 Andrei Leitão,3 Tudor I. Oprea,2 Bin Chen,1 Teresa Louis,1 Anne Marie Bertucci,1 Catherine G.N. Sharma,2 Shaun D. Gill,2 Helen R. Kim,2 Heather A. Shupp,2 Jennifer R. Pyle,2 Alexis Madrack,2 Anne L. Donato,2 Dong Cheng,1 James R. Paige,1 and V. Craig Jordan1,2

1Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, Illinois; 2Fox Chase Cancer Center, Philadelphia, Pennsylvania; and 3Division of Biocomputing, University of New Mexico Health Sciences Center, Albuquerque, New Mexico

Abstract

Aromatase inhibitors (AI) are being evaluated as long-term adjuvant therapies and chemopreventives in breast cancer. However, there are concerns about bone mineral density loss in an estrogen-free environment. Unlike nonsteroidal AIs, the steroidal AI exemestane may exert beneficial effects on bone through its primary metabolite 17-hydroxexemestane. We investigated 17-hydroxexemestane and observed it bound estrogen receptor α (ERα) very weakly and androgen receptor (AR) strongly. Next, we evaluated 17-hydroxexemestane in MCF-7 and T47D breast cancer cells and attributed dependency of its effects on ER or AR using the antiestrogen fulvestrant or the antiandrogen bicalutamide. 17-Hydroxexemestane induced proliferation, stimulated cell cycle progression and regulated transcription at high sub-micromolar and micromolar concentrations through ER in both cell lines, but through AR at low nanomolar concentrations selectively in T47D cells. Responses of each cell type to high and low concentrations of the non-aromatizable synthetic androgen R1881 paralleled those of 17-hydroxexemestane. 17-Hydroxexemestane down-regulated ERα protein levels at high concentrations in a cell type–specific manner similarly as 17β-estradiol, and increased AR protein accumulation at low concentrations in both cell types similarly as R1881. Computer docking indicated that the 17β-OH group of 17-hydroxexemestane relative to the 17-keto group of exemestane contributed significantly more intermolecular interaction energy toward binding AR than ERα. Molecular modeling also indicated that 17-hydroxexemestane interacted with ERα and AR through selective recognition motifs employed by 17β-estradiol and R1881, respectively. We conclude that 17-hydroxexemestane exerts biological effects as an androgen. These results may have important implications for long-term maintenance of patients with AIs. [Mol Cancer Ther 2007;6(11):2817–27]

Introduction

The third-generation aromatase inhibitors (AI) anastrozole (Arimidex; refs. 1, 2), letrozole (Femara; refs. 3, 4), and exemestane (Aromasin; refs. 5, 6), by virtue of blocking extragonadal conversion of androgens to estrogens and giving rise to an estrogen-depleted environment, exhibit improved efficacy over tamoxifen in the adjuvant therapy of estrogen receptor (ER)–positive breast cancer in post-menopausal women (7). Clinical trials evaluating these AIs showed a reduced incidence of contralateral primary breast cancer in the AI groups compared with tamoxifen (1–6); hence, AIs are currently being evaluated as chemopreventives in ongoing studies (8). AIs also exhibit reduced overall toxicity compared with tamoxifen (1–6, 9), but the toxicity profiles are different: tamoxifen is associated with increased incidences of thromboembolic events and endometrial cancer, whereas AIs are associated with decreased bone mineral density (BMD), coupled with an increased risk of bone fractures (10–12) and severe musculoskeletal pain that limits patient compliance (13, 14). Because the available third-generation AIs all exhibit similar efficacies, the selection of a specific AI for long-term adjuvant therapy of breast cancer and as a chemopreventive in healthy women at high risk for breast cancer will likely be determined by safety and tolerability profiles.

AIs fall into two classes, steroidal as represented by exemestane, which acts as a suicide inhibitor of aromatase, and nonsteroidal including anastrozole and letrozole, which reversibly block aromatase activity (7). Possibly androgen. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 5/3/07; revised 8/28/07; accepted 10/1/07.
Grant support: Department of Defense Breast Program under award BC050277 Center of Excellence (V.C. Jordan; views and opinions of, and endorsements by the author(s) do not reflect those of the U.S. Army or the Department of Defense), Specialized Programs of Research Excellence in Breast Cancer CA89018 (V.C. Jordan), the Avon Foundation (V.C. Jordan), the Weg Fund (V.C. Jordan), and NIH P30 CA006927 (Fox Chase Cancer Center), an Eli Lilly Fellowship (Robert H. Lurie Comprehensive Cancer Center), the Lynn Sage Breast Cancer Research Foundation (Robert H. Lurie Comprehensive Cancer Center), the NIH Molecular Libraries Initiative award US4 MH074425-01, and by National Cancer Institute CA118100 (University of New Mexico Cancer Center).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: V. Craig Jordan, Alfred G. Knudson Chair of Cancer Research, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111-2497. Phone: 215-728-7410; Fax: 215-728-7034. E-mail: v.craig.jordan@fccc.edu
Copyright © 2007 American Association for Cancer Research. doi:10.1158/1535-7163.MCT-07-0312

Mol Cancer Ther 2007;6(11):2817–27
Published OnlineFirst November 7, 2007; DOI: 10.1158/1535-7163.MCT-07-0312
Downloaded from mct.aacrjournals.org on April 19, 2017. © 2007 American Association for Cancer Research.
of osteoporosis, and found to reduce bone resorption markers and increase BMD and bone strength, whereas lowering serum cholesterol and low-density lipoprotein levels compared with ovariectomized controls. One of these preclinical studies also evaluated the nonsteroidal AI letrozole, but in contrast, found no benefit of letrozole on bone or lipid profiles (16). In a clinical study investigating the effects of 2 years of exemestane on bone compared with placebo without prior tamoxifen therapy in patients with surgically resected breast cancer at low risk for recurrence, exemestane did not enhance BMD loss in lumbar spine and only modestly enhanced BMD loss in the femoral neck compared with the placebo group (17). Interestingly, in this study, exemestane promoted bone metabolism by increasing levels of both bone resorption and formation markers (17). However, a clear-cut advantage of exemestane versus the nonsteroidal AIs on bone safety has not been shown in humans, possibly because all other clinical studies compared the AI to tamoxifen (9, 12, 18) or the AI to placebo with prior tamoxifen therapy (10, 11). Drawing conclusions from these studies is difficult because tamoxifen preserves BMD, thereby protecting against fractures, and withdrawal of tamoxifen may have lasting effects on BMD (19).

Maintenance of BMD in women is a known estrogenic effect (20). However, androgen receptors (AR) are also expressed in multiple bone cell types (21, 22), and studies show that androgens maintain BMD in ovariectomized rats (23, 24) and in women (21, 25–27). In ovariectomized rats, physiologic concentrations of androstenedione, a weak androgen and a substrate of aromatase, reduced loss of bone, and the antiandrogen bicalutamide abrogated this effect (23), but anastrozole did not (23). Therefore, the protective effect of androstenedione on maintenance of BMD was androgen mediated and not due to aromatization of androstenedione to estrogen. Furthermore, the nonaromatizable androgen 5α-dihydrotestosterone has been shown to stimulate bone growth in osteoporotic ovariectomized rats (24). In pre- and postmenopausal women, endogenous androgen levels correlate with BMD (25, 26). Furthermore, a study comparing estrogen to a synthetic androgen in postmenopausal osteoporotic women showed that both steroids were equally effective in reducing bone resorption (27). Also, a 2-year double-blind trial showed that estrogen plus a non-aromatizable androgen significantly improved BMD over estrogen alone in surgically menopausal women (28). Therefore, exogenous androgens promote BMD maintenance in women when used alone (27) and in conjunction with estrogen (28).

Although exemestane does not bind ER, it is structurally related to androstenedione and has weak affinity for AR (29, 30). At high doses, exemestane exerts possible androgenic activity in vivo by inducing an increase in ventral prostate weight in immature castrated rats (29). Recently, Miki et al. (22) showed in human osteoblast hFOB and osteosarcoma Saos-2 cells that exemestane promoted proliferation, which was partially blocked by the antiandrogen hydroxyflutamide, and increased alkaline phosphatase activity. However, metabolites of exemestane may be mediating these effects. Exemestane is given p.o. at 25 mg/day and rapidly absorbed, showing peak plasma levels within 2 to 4 h and a direct relationship between dosage and peak plasma levels after single (10–200 mg) or repeated doses (0.5–50 mg; refs. 30, 31). Single-dose studies suggested that exemestane has a short elimination half-life, but multiple-dose studies show its terminal half-life to be about 24 h. Exemestane undergoes complex metabolism, and the primary metabolite in plasma has been identified as 17-hydroexemestane, which accumulates to a concentration of about 10% of its parent compound (30). Taking the possible action of metabolites into consideration, Goss et al. (16) administered 17-hydroexemestane to ovariectomized rats and found that it produced the same bone-sparing effects and favorable changes in circulating lipid levels as exemestane. Also, Miki et al. (22) stated that 17-hydroexemestane promoted proliferation of the osteoblast and osteosarcoma cells similar to exemestane, but the data were not shown, and the authors did not further explore 17-hydroexemestane activities. Additionally, Miki et al. (22) showed that the osteoblasts efficiently metabolized androstenedione to testosterone, which involves the reduction of the 17-keto group of androstenedione to a hydroxyl group. Similar metabolism would convert exemestane to 17-hydroexemestane, and thus, activities of exemestane in the osteoblasts may have been mediated by a metabolite of exemestane. Hence, a thorough investigation of exemestane and 17-hydroexemestane activities through ER and AR is warranted to provide evidence regarding whether exemestane could display a more favorable safety and toxicity profile than nonsteroidal AIs for long-term adjuvant use and as a chemopreventive of breast cancer in postmenopausal women. Therefore, we evaluated the pharmacologic actions of exemestane and its primary metabolite 17-hydroexemestane on ER- and AR-regulated activities in a range of cellular and molecular assays. First, we determined the relative binding affinity (RBA) of 17-hydroexemestane to ERα and ERβ. Next, using MCF-7 and T47D breast cancer cells, we examined the ability of 17-hydroexemestane to stimulate cell proliferation and cell cycle progression (Supplementary Material) via ER and AR, to regulate ER- and AR-dependent transcription, and to modulate ERα and AR protein levels. Lastly, we investigated intermolecular interactions between 17-hydroexemestane and ERα and AR using molecular modeling.

Materials and Methods

Compounds and Cell Lines

Exemestane and 17-hydroexemestane were provided by Pfizer. Fulvestrant (ICI 182,780, Faslodex) and bicalutamide (Casodex) were provided by Dr. Alan E. Wakeling and Dr. Barrington J.A. Furr (AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom), respectively. All other

4 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
compounds were obtained from Sigma-Aldrich, and cell culture reagents were from Invitrogen. All test agents were dissolved in ethanol and added to the medium at 1:1,000 (v/v). MCF-7/wS8 and T47D:A18 human mammary carcinoma cells, clonally selected from their parental counterparts for sensitivity to growth stimulation by E2 (32), were used in all experiments indicating MCF-7 and T47D cells. Cells were maintained in steroid-replete RPMI 1640, but 3 days before all experiments, were cultured in steroid-free media as previously described (32, 33).

**Competitive Hormone-Binding Assays**

Competitive hormone-binding assays were conducted using fluorescence polarization–based ERs and AR Competitor Assay kits (Invitrogen) as previously described (34).

**Cellular Proliferation Assays**

Cellular proliferation following 7 days in culture was determined by DNA mass per well in 12-well plates using the fluorescent DNA dye Hoechst 33258 as previously described (32).

**Reporter Gene Assays**

Reporter gene assays were conducted by transfecting cells with either an ERE(5x)-regulated (pERE(5x)TA-ffLuc; ref. 33) or ARE(5x)-regulated (pAR-Luc; Panomics) firefly luciferase expression plasmid and co-transfected with a basal TATA promoter-regulated (pTA-srLuc) Renilla luciferase expression plasmid as previously described (33).

**Quantitative Real-Time PCR**

Quantitative real-time PCR (qPCR) was used to determine AR and ribosomal large phosphoprotein subunit P0 (RLP0; 36B4) mRNA levels as previously described (35).

**Immunoblot Analyses**

Immunoblots, prepared as previously described (33), were probed with primary antibodies against AR (AR 441; Lab Vision), ERα (AER 611; Lab Vision), and β-actin (AC-15; Sigma-Aldrich).

**Molecular Modeling and Virtual Docking Calculations**

The three-dimensional conformations for E2, 17-hydroexemestane, exemestane, R1881, and dexamethasone were generated with Omega version 2.1 software (OpenEye Scientific Software). These compounds were docked using the following X-ray crystallographic structures: 1GWR (ERα co-complexed with E2, 2.4-Å resolution; ref. 36) and 1XQ3 (AR co-complexed with R1881, 2.25-Å resolution; ref. 37). ERα and AR ligand-binding pockets were built using a ligand-centered box and the receptor-bound conformation of the respective ligand: E2 (for 1GWR) and R1881 (for 1XQ3). The volume of the cavity differs for the two receptors: 648 Å³ for 1GWR and 532 Å³ for 1XQ3. All receptor and ligand bonds were kept rigid. The receptor structures were filled with water because ERα (38) and AR crystal structures (39) indicate that specific stable hydrogen bond (H-bond) networks form among particular water molecules, ligands, and amino acid side chains. Docking was done with FRED version 2.2 software (OpenEye) using a short refinement step for the ligands within the receptor and using the MMFF94 force field. The best 30 conformations for each compound were compared and ranked by FRED’s Chemscore function. For each ligand-docked receptor evaluated, the docked conformation with the lowest total intermolecular interaction energy (kJ/mol) was selected. To address whether water could be displaced by a compound during the process of binding, docking calculations were also done using receptors modeled with water removed as presented in Supplementary Table S1 and the differences between the methods in Supplementary Table S2.

**Curve Fitting and Statistical Analyses**

All statistical tests, curve fitting, and determination of half-maximal inhibitory concentrations (IC₅₀) and half-maximal effective concentrations (EC₅₀) were done using GraphPad Prism 4.03 (GraphPad Software). Significant differences were determined using one-way ANOVA with Bonferroni multiple comparison post-test.

**Results**

**Experimentally Determined Binding of 17-Hydroexemestane and Exemestane to ERα and AR**

Structures of the compounds relevant to these studies, the steroidal AI parent compound exemestane, its primary metabolite 17-hydroexemestane, E₂, and the synthetic non-aromatizable androgen R1881, are shown in Fig. 1A. Importantly, the only difference between parental exemestane and its metabolite 17-hydroexemestane is a hydroxyl group in the metabolite in place of a ketone in the parent compound at the 17β position, whereas both compounds share a 3-keto group. For steroidal estrogens, elimination or modification of the 17β-OH group reduces binding to ERα, but that of the 3-OH group is much more dramatic (40). For steroidal androgens, the trend is reversed; elimination or modification of the 17β-OH group is more significant for AR binding than that of the 3-keto group (41). The 3-keto group found in both exemestane and 17-hydroxexemestane also favors binding to AR (41).

We tested the binding of exemestane and 17-hydroxexemestane to ERα and AR using fluorescence polarization–based competitive hormone-binding assays (Fig. 1B and C; Table 1). For purposes of comparison, compound affinities were arbitrarily categorized with respect to their RBAs as strong (100 to ≥1), moderate (<1 to ≥0.1), weak (<0.1 to ≥0.01), or very weak (<0.01 to detectable binding defined as 50% competition), and inactive (compound did not compete for at least 50% binding). E₂ competitively bound ERα with an IC₅₀ of 1.33 × 10⁻⁶ mol/L (RBA = 100; Fig. 1B), and R1881 competitively bound AR with an IC₅₀ of 1.34 × 10⁻⁶ mol/L (RBA = 100; Fig. 1C). Considering ERα (Fig. 1B), both R1881 and 17-hydroxexemestane competed for binding to ERα with IC₅₀ of 1.02 × 10⁻⁶ mol/L (RBA = 0.130) and 2.12 × 10⁻⁶ mol/L (RBA = 0.006), respectively, which categorized R1881 as a moderate and 17-hydroxexemestane as a very weak ERα ligand. Neither exemestane nor dexamethasone significantly competed for binding to ERα. Regarding AR (Fig. 1C), 17-hydroxexemestane and exemestane competed for binding to AR with IC₅₀ of 3.96 × 10⁻⁸ mol/L (RBA = 33.8) and 2.03 × 10⁻⁸ mol/L (RBA = 0.658), respectively, which classified
Androgenic and Estrogenic Effects of 17-Hydroexemestane

Proliferation Responses to 17-Hydroexemestane and Exemestane

We examined the effects of exemestane and 17-hydroexemestane on 7 days of proliferation in ERα- and AR-positive MCF-7 and T47D mammary carcinoma cells (Fig. 2). As expected, both cell lines were growth stimulated by E2, with growth EC50s of $1.7 \times 10^{-12}$ mol/L E2 for MCF-7 cells (Fig. 2A) and $7.1 \times 10^{-12}$ mol/L E2 for T47D cells (Fig. 2B). These growth responses to E2 were completely blocked by fulvestrant (all $P$ values <0.001), validating the $E_2$ responsiveness via ER in these cell lines.

Both cell lines were also growth stimulated by R1881 (Fig. 2A and B) and 17-hydroexemestane (Fig. 2C and D), whereas exemestane did not exert any significant effect on proliferation (Fig. 2C and D). Considering MCF-7 cells, R1881 exhibited a growth EC50 of $2.4 \times 10^{-8}$ mol/L (Fig. 2A), or approximately 4 orders of magnitude higher than that of E2. Similarly, 17-hydroexemestane exhibited a growth EC50 of $2.7 \times 10^{-6}$ mol/L in MCF-7 cells (Fig. 2C) or approximately 6 orders of magnitude higher than that of E2. These growth responses to R1881 and 17-hydroexemestane in MCF-7 cells were completely blocked by cotreatment with fulvestrant (Fig. 2A and B; both $P$ values <0.001). Therefore, whereas R1881, a non-aromatizable synthetic androgen, stimulated growth of MCF-7 cells, it did so by acting through ER. Hence, at high concentrations, R1881 exerted estrogenic activity. Similarly, at high concentrations, 17-hydroexemestane also exerted estrogenic activity and stimulated growth of MCF-7 cells by acting through ER.

Interestingly, in T47D cells, the growth response to R1881 and 17-hydroexemestane followed an apparent bimodal pattern, which was different than in MCF-7 cells. In T47D cells, proliferative effects of high concentrations of R1881 ($5 \times 10^{-6}$ mol/L; Fig. 2B) and 17-hydroexemestane ($5 \times 10^{-6}$ mol/L; Fig. 2D) were only partially blocked by fulvestrant (both $P$ values <0.001), down to the level of growth observed at nanomolar concentrations of these compounds. However, proliferative effects of lower concentrations of R1881 ($10^{-9}$ mol/L) and 17-hydroexemestane ($10^{-8}$ mol/L) were completely blocked by the anti-androgen bicalutamide (both $P$ values <0.001). Based on these observed levels of inhibition by bicalutamide and fulvestrant, maximal concentrations at which R1881 and 17-hydroexemestane stimulated growth through AR-dependent activities were $10^{-7}$ and $10^{-6}$ mol/L, respectively, and above these concentrations, R1881 and 17-hydroexemestane stimulated growth through ER-dependent activities. Using this information to define concentration ranges in which these compounds exert AR-mediated or ER-mediated effects in T47D cells, the growth EC50s via AR of R1881 and 17-hydroexemestane were 1.0 x $10^{-10}$ mol/L (Fig. 2B) and 4.3 x $10^{-10}$ mol/L (Fig. 2D), respectively. Similarly, the growth EC50s via ER of R1881 and 17-hydroexemestane in T47D cells were 3.1 x $10^{-7}$ mol/L (Fig. 2B) and 1.5 x $10^{-6}$ mol/L (Fig. 2D), respectively. Hence, in T47D cells, both R1881 and 17-hydroexemestane stimulated growth via AR at lower concentrations, whereas both compounds stimulated growth through ER at higher concentrations.

Figure 1. Compounds examined in this study and their RBAs for ERα and AR. A, structures of exemestane, its primary metabolite 17-hydroexemestane E2, and R1881. ERα (B) and AR (C) fluorescence polarization-based competitive hormone-binding assays. Baculovirus-produced human ERα and rat AR ligand-binding domain tagged with a His-glutathione S-transferase epitope (His-GST-ARLBD) were used at final concentrations of 15 and 25 nmol/L, respectively. The fluorescently labeled E2α and AR ligands, Fluormone ES2 and Fluormone AL Green, respectively, were both used at a final concentration of 1 nmol/L. The competing test compounds were E2, R1881, 17-hydroexemestane, exemestane, and dexamethasone (DEX) as indicated. Point, mean of triplicate determinations; bars, 95% confidence intervals. Curve fitting was done using GraphPad Prism software (version 4.03). IC50s corresponding to a half-maximal shift in polarization values of the test compounds were determined using the maximum and minimum polarization values of the E2α-competitive binding curve for ERα or of the R1881-competitive binding curve for AR as appropriate.

17-hydroexemestane as a strong and exemestane as a weak AR ligand. However, dexamethasone would also be categorized as a weak AR ligand. Hence, the observed very weak ERα binding and strong AR binding of 17-hydroexemestane was consistent with what previously reported structure-activity relationships (40, 41) would have predicted due to reduction of the 17-keto group in exemestane to a 17β-OH in the metabolite.
concentrations and via ER at higher concentrations. These results were consistent with the observed binding affinities of these compounds to ERs (Fig. 1B) and AR (Fig. 1C).

Cell Cycle Progression Responses to 17-Hydroxexemestane

As shown in Supplementary Fig. S1, 17-hydroxexemestane at 10\(^{-8}\) mol/L acted through AR to stimulate S-phase entry in T47D cells by 1.9-fold (P < 0.001) but, at 5 \times 10^{-6} mol/L, acted through ER to stimulate S-phase entry in MCF-7 cells by 2.2-fold (P < 0.001). Hence, 17-hydroxexemestane effects on cell cycle progression were consistent with its effects on proliferation (Fig. 2).

Regulation of ERs and AR Transcriptional Activities by 17-Hydroxexemestane

Next, we investigated the ability of 17-hydroxexemestane to regulate ER and AR transcriptional activity by transfecting cells with an ERE(5x)-regulated or ARE(5x)-regulated dual-luciferase plasmid set, treating cells with these compounds, and measuring dual-luciferase activity 44 h after treatment (Fig. 3A–C). E2 at 10\(^{-10}\) mol/L induced transcriptional activity was blocked by fulvestrant 44 h after treatment (Fig. 3A). At high sub-micromolar concentrations, R1881 stimulated ERE(5x)-regulated transcription. At high sub-micromolar concentrations, R1881 acted as an estrogen. In a similar manner, AR-dependent transcriptional activity was investigated. T47D cells showed a concentration-dependent induction of ARE(5x)-regulated transcription in response to R1881, with 10\(^{-9}\) mol/L R1881 inducing transcription by 8.5-fold and 10\(^{-6}\) mol/L R1881 maximally inducing transcription by 12.7-fold relative to control-treated cells (Fig. 3C; both P values <0.001). Bicalutamide blocked 10\(^{-9}\) mol/L R1881-mediated induction of ARE(5x)-regulated transcription (Fig. 3C; P < 0.001), confirming dependence on AR. MCF-7 cells failed to respond to 10\(^{-6}\) mol/L R1881 with induction of ARE(5x)-regulated transcription (data not shown), although these cells express AR protein. This supports our prior results that T47D cells were growth stimulated by R1881 through an AR-dependent mechanism (Fig. 2B), but that MCF-7 cells were not (Fig. 2A). As expected, 10\(^{-6}\) mol/L E2 failed to induce ARE(5x)-regulated transcription (Fig. 3C). Next, 17-hydroxexemestane was evaluated in T47D cells and, in a concentration-dependent manner, induced ARE(5x)-regulated transcription with maximal induction of 4.7-fold occurring at 5 \times 10^{-6} mol/L relative to control treatment (Fig. 3C; P < 0.001). However, because high concentrations of 17-hydroxexemestane were needed to induce this synthetic ARE(5x)-regulated promoter, we tested whether lower concentrations of 17-hydroxexemestane could modulate endogenous AR mRNA expression, which is known to be negatively feedback regulated by its gene product (42). Using real-time PCR, AR mRNA levels were determined in T47D cells following 24 h of treatment with test compounds (Fig. 3D). R1881 at 10\(^{-9}\) mol/L significantly down-regulated

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Receptor</th>
<th>Competitive hormone binding</th>
<th>Intermolecular interaction energy (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(IC(_{50}) (mol/L)</td>
<td>95% CI (mol/L)</td>
</tr>
<tr>
<td>E2</td>
<td>ERs</td>
<td>1.33 \times 10^{-9}</td>
<td>1.18–1.49 \times 10^{-9}</td>
</tr>
<tr>
<td>R1881</td>
<td>ERs</td>
<td>1.02 \times 10^{-6}</td>
<td>0.90–1.15 \times 10^{-6}</td>
</tr>
<tr>
<td>17-Hydroxexemestane</td>
<td>ERs</td>
<td>2.12 \times 10^{-5}</td>
<td>1.73–2.61 \times 10^{-5}</td>
</tr>
<tr>
<td>Exemestane</td>
<td>ERs</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>ERs</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>R1881</td>
<td>AR</td>
<td>1.34 \times 10^{-8}</td>
<td>1.00–1.79 \times 10^{-8}</td>
</tr>
<tr>
<td>17-Hydroxexemestane</td>
<td>AR</td>
<td>3.96 \times 10^{-8}</td>
<td>2.74–5.71 \times 10^{-8}</td>
</tr>
<tr>
<td>Exemestane</td>
<td>AR</td>
<td>2.03 \times 10^{-5}</td>
<td>1.39–2.97 \times 10^{-5}</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>AR</td>
<td>1.03 \times 10^{-5}</td>
<td>0.75–1.43 \times 10^{-5}</td>
</tr>
</tbody>
</table>

Abbreviations: RTB Penalty, rotatable bond penalty; NA, not applicable; test compound did not compete for at least 50% binding of ERs.
AR mRNA expression by 48% (P < 0.001), whereas 10⁻⁸ mol/L E₂ did not (Fig. 3D). Bicalutamide prevented R1881-mediated decrease in AR mRNA expression (Fig. 3D), validating that AR mRNA levels were negatively feedback regulated. Similarly, a low 10⁻⁸ mol/L concentration of 17-hydroexemestane led to a 41% decrease in AR mRNA levels (P < 0.01), with increased 17-hydroexemestane concentrations further decreasing AR mRNA expression (Fig. 3D). Bicalutamide blocked 17-hydroexemestane-mediated down-regulation of AR mRNA expression (P < 0.01), whereas fulvestrant did not (Fig. 3D). Therefore, 17-hydroexemestane acted as an androgen via AR to feedback-regulate the expression of endogenous AR mRNA in T47D cells.

**Modulation of AR and ERα Protein Levels by 17-Hydroexemestane**

Androgens and estrogens modulate protein expression levels of their cognate receptors. R1881 stabilizes AR protein allowing its accumulation (43), whereas E₂ promotes ERα degradation in a cell type−dependent manner (32). Therefore, we investigated the effects of 17-hydroexemestane on AR and ERα protein levels by treating cells with test compounds for 24 h and analyzing receptor levels by immunoblotting. E₂ did not significantly affect AR protein accumulation in MCF-7 cells.
(Fig. 4A), but did down-regulate AR protein levels in T47D cells (Fig. 4B). Also, fulvestrant and E2 plus fulvestrant treatments did not significantly affect AR protein levels in MCF-7 cells (Fig. 4A), but did modestly up-regulate AR protein levels in T47D cells (Fig. 4B). As expected, R1881 caused an increase in accumulation of AR protein in both cell lines (Fig. 4A and B), likely by stabilizing the protein (43). Next, we characterized the effects of low $10^{-8}$ mol/L and high $5 \times 10^{-6}$ mol/L concentrations of 17-hydroxexemestane on ERα and AR expression. The high $5 \times 10^{-6}$ mol/L concentration of 17-hydroxexemestane led to decreased ERα protein levels in MCF-7 (Fig. 4A), but not in T47D cells (Fig. 4B); this pattern indicates that $5 \times 10^{-6}$ mol/L 17-hydroxexemestane acted as an estrogen to regulate ERα protein in a cell type–dependent manner. Similar to R1881, treatment with low $10^{-8}$ mol/L or high $5 \times 10^{-6}$ mol/L concentrations of 17-hydroxexemestane led to increased AR protein accumulation in both cell lines (Fig. 4A and B), indicating that 17-hydroxexemestane acted as an androgen likely by stabilizing AR protein. Therefore, 17-hydroxexemestane-modulated ERα and AR protein accumulation as would an estrogen and an androgen, respectively.

**Molecular Docking of 17-Hydroxexemestane and Exemestane to ERα and AR**

To investigate the mechanism by which 17-hydroxexemestane binds ERα as a very weak ligand and AR as a

![Image](image_url)

**Figure 3.** 17-Hydroxexemestane and R1881 regulate ER transcriptional activity at high concentrations and AR transcriptional activity at low concentrations. ERE(5x)-regulated dual-luciferase activity in (A) MCF-7 cells and (B) T47D cells. (C) ARE(5x)-regulated reporter gene activity in T47D cells. A–C, Under steroid-free conditions, cells were transiently transfected with pERE(5x)-TA-ffLuc or pARE(5x)-Luc (firefly luciferase reporter plasmids) and the internal normalization control pTA-srLuc (Renilla luciferase reporter plasmid). Four hours after transfection, cells were treated as indicated and then again the following day. Cells were assayed 44 h after transfection for dual-luciferase activity. Data shown are the mean of triplicate determinations and associated SDs. 17-Hydroxexemestane and R881 stimulated ERE(5x)-regulated transcription in MCF-7 and T47D cells and ARE(5x)-regulated transcription in T47D cells. D, AR mRNA levels in T47D cells as determined by real-time PCR. T47D cells were treated as indicated for 24 h. RNA was isolated and converted to cDNA. Continuous accumulation of PCR products was monitored using the double strand-specific DNA dye SYBR Green. Quantitative measurements of AR mRNA and the endogenous normalization control RLP0 mRNA were determined by comparison to a standard curve of known quantities of serially diluted AR or RLP0 PCR product. The data represent the mean and SDs of three independent samples, each of which was measured in triplicate. 17-Hydroxexemestane and R881 down-regulated AR mRNA levels at nanomolar concentrations in an AR-dependent manner.
Androgenic and Estrogenic Effects of 17-Hydroexemestane

2824

Figure 4. 17-Hydroexemestane modulates AR and ERα protein levels. Immunoblot analysis of AR and ERα in (A) MCF-7 cells and (B) T47D cells. Cells were treated as indicated for 24 h, and 20 μg of cellular protein were resolved by 4% to 12% SDS-PAGE and then transferred to a nylon membrane. Membranes were probed for AR, ERα, and β-actin, and immunoreactive bands were visualized by chemiluminescence and autoradiography. Cropped blots are shown. 17-hydroexemestane up-regulated AR protein levels at 10^−9 mol/L in both cell lines and down-regulated ERα in MCF-7 cells at 5 × 10^−9 mol/L.

Discussion

We observed that 17-hydroexemestane, the primary metabolite of exemestane, bound to ERα as a very weak ligand and acted through ERα at high sub-micromolar and micromolar concentrations to stimulate growth, promote cell cycle progression, induce ERE-regulated reporter gene expression, and down-modulate ERα protein levels in breast cancer cells. However, we also observed that 17-hydroexemestane bound to AR as a strong ligand and found in T47D cells that 17-hydroexemestane stimulated growth, induced cell cycle progression, down-modulated AR mRNA expression, and stabilized AR protein levels, with all of these effects occurring at low nanomolar concentrations and blocked by bicalutamide. Moreover, computer docking indicated that the 17β-OH group of 17-hydroexemestane versus the 17-keto group of exemestane contributed significantly more toward increasing affinity to AR than to ERα. Molecular modeling also indicated that 17β-OH group of 17-hydroexemestane interacted with AR through an important H-bond of Asn705, a conserved recognition motif employed by R1881. Therefore, we propose that the primary mechanism of action of exemestane in vivo is mediated by 17-hydroexemestane regulating AR activities.

The Food and Drug Administration label for exemestane (Aromasin; Pfizer) reports that in postmenopausal women with advanced breast cancer, the mean AUC (area under the curve) values of exemestane following repeated doses was 75.4 ng·h/mL (254 nmol·h/L), which was almost twice that in healthy postmenopausal women (41.4 ng·h/mL; 140 nmol·h/L; ref. 31). Because circulating levels of 17-hydroexemestane can reach about 1/10 the level of the parent compound (30), we hypothesize that circulating levels of 17-hydroexemestane are sufficient to bind AR and

and decreased steric clash by −1.08 kJ/mol. Hence, the 17β-OH group in 17-hydroexemestane compared with the 17-keto group in exemestane contributed −5.47 kJ/mol toward higher affinity for binding AR (Table 1). In the R1881 docked to AR model, H-bonds between R1881 and Asn705, Gln711 and Arg792 were observed (Fig. 5B). The OH side chain of Thr777 was in close proximity to both docked R1881 (Fig. 5B) and 17-hydroexemestane (Fig. 5D), but the angle was not favorable for H-bonding. Docking of 17-hydroexemestane to AR (Fig. 5D) indicated a short 2.78-Å H-bond between the 17β-OH group of the ligand and Asn705 but not between the 3-keto group of the ligand and Gln711 and Arg792. Hence, the short 2.78-Å H-bond observed in the 17-hydroexemestane docked to AR model was important in mediating high affinity binding. The exemestane docked to AR model is shown in Supplementary Fig. S2B.4

Considering ERα, the intermolecular interaction energies of R1881 and 17-hydroexemestane were less favorable than E2 by 1.94 and 2.76 kJ/mol, respectively, due to decreased interaction energies of the crystal structures. Superimposition of the docked and crystallo-
graphic structures of E2 complexed with ERα (Fig. 5A) and of R1881 complexed with AR (Fig. 5B) showed that the docking models recapitulated the molecular recognition patterns of the crystal structures.

Considering ERα, the intermolecular interaction energies of R1881 and 17-hydroexemestane were less favorable than E2 by 1.94 and 2.76 kJ/mol, respectively, due to decreased H-bond interactions and increased steric clash (Table 1). Exemestane was much less favorable than E2 by 4.57 kJ/mol (Table 1). Hence, the 17β-OH group of 17-hydroexemestane compared with the 17-keto group of exemestane contributed −1.81 kJ/mol toward increased affinity for ERα. Interestingly, the docking calculations suggested that the higher affinity of 17-hydroexemestane over exemestane to ERα was not due to increased H-bonding mediated by the 17β-OH group, but rather increased lipophilic interactions (Table 1) due to a slight repositioning of the compound as a consequence of 17β-OH group. In the E2 docked to ERα model, H-bonds between E2 and Glu353, Arg394, and His524 side chains were observed (Fig. 5A). In the docked 17-hydroexemestane to ERα model (Fig. 5C), the same Arg394 and His524 interactions were maintained, except that there was a loss of the Glu353 interaction. The R1881 docked to ERα model is shown in Supplementary Fig. S2A.4

Considering AR, the intermolecular interaction energy of 17-hydroexemestane was only 0.8 kJ/mol less favorable than R1881, whereas exemestane was significantly less favorable than R1881 by 6.27 kJ/mol (Table 1). Docking of 17-hydroexemestane to AR, compared with the parent drug exemestane, indicated that 17-hydroexemestane exhibited improved lipophilic interactions by −2.11 kJ/mol, more favorable H-bonding interactions by −2.65 kJ/mol, and decreased steric clash by −1.08 kJ/mol. Hence, the 17β-OH group in 17-hydroexemestane compared with the 17-keto group in exemestane contributed −5.47 kJ/mol toward higher affinity for binding AR (Table 1). In the R1881 docked to AR model, H-bonds between R1881 and Asn705, Gln711 and Arg792 were observed (Fig. 5B). The OH side chain of Thr777 was in close proximity to both docked R1881 (Fig. 5B) and 17-hydroexemestane (Fig. 5D), but the angle was not favorable for H-bonding. Docking of 17-hydroexemestane to AR (Fig. 5D) indicated a short 2.78-Å H-bond between the 17β-OH group of the ligand and Asn705 but not between the 3-keto group of the ligand and Gln711 and Arg792. Hence, the short 2.78-Å H-bond observed in the 17-hydroexemestane docked to AR model was important in mediating high affinity binding. The exemestane docked to AR model is shown in Supplementary Fig. S2B.4

Figure 4. 17-Hydroexemestane modulates AR and ERα protein levels. Immunoblot analysis of AR and ERα in (A) MCF-7 cells and (B) T47D cells. Cells were treated as indicated for 24 h, and 20 μg of cellular protein were resolved by 4% to 12% SDS-PAGE and then transferred to a nylon membrane. Membranes were probed for AR, ERα, and β-actin, and immunoreactive bands were visualized by chemiluminescence and autoradiography. Cropped blots are shown. 17-hydroexemestane up-regulated AR protein levels at 10^−9 mol/L in both cell lines and down-regulated ERα in MCF-7 cells at 5 × 10^−9 mol/L.
regulate AR-dependent activities. Furthermore, a subpopulation of patients may exist who metabolize exemestane at higher rates, leading to correspondingly higher circulating 17-hydroexemestane levels. For instance, one of three patients administered 800 mg of exemestane, the highest dose evaluated, achieved 17-hydroexemestane plasma levels approximately one-half the level of the parent compound (30). Based on our results, we would predict that higher circulating levels of 17-hydroexemestane would associate with decreased rates of BMD loss and risk of bone fractures in postmenopausal women. We suggest that circulating levels of 17-hydroexemestane and exemestane should be determined in clinical trials and correlated to disease outcome and toxicity profiles such as BMD loss.

Although the clinical studies reported thus far were not designed to directly compare one AI versus another, comparisons in the rate of BMD loss from baseline to year 1, and from year 1 to 2 can be made. In the bone safety subprotocol of the IES (Intergroup Exemestane Study) trial, the rate of BMD loss was greatest within 6 months of switching from tamoxifen to exemestane at -2.7% in the lumbar spine and -1.4% in the hip, but thereafter, BMD loss progressively slowed in months 6 to 12 and again in months 12 to 24 to only -1.0% and -0.8% in the lumbar spine and hip, respectively (10), which is in the same range as would be expected for postmenopausal women in general. However, in the bone safety substudy of the MA.17 trial, patients administered letrozole experienced a relatively constant rate of BMD loss for 2 years: at 12 months, the rate of BMD loss from baseline was -3.3% and -1.43% in lumbar spine and hip, respectively, and from year 1 to year 2, -2.05% and -2.17% in lumbar spine and hip, respectively (11). In the bone substudy of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial, the rate of BMD loss from baseline to year 1 was -2.2% in lumbar spine and -1.5% in hip and from year 1 to year 2, -1.8% in lumbar spine and -1.9% in hip (18). Collectively, these results suggest that after the initial

![Figure 5](image-url)
Androgenic and Estrogenic Effects of 17-Hydroexemestane

12 months of AI therapy, exemestane may be associated with slower rates of BMD loss compared with nonsteroidal AIs. Furthermore, although not directly comparable, the fracture rate per 1,000 woman-years in the ATAC trial was 22.6 for anastrozole and 15.6 for tamoxifen (1), whereas in the IES trial, the incidence rate per 1,000 woman-years for multiple fractures was 19.2 for exemestane and 15.1 for tamoxifen (10). These results show that although both anastrozole and exemestane were associated with higher fracture rates than tamoxifen, they also suggest that exemestane may be associated with a lower fracture rate than anastrozole. Clinical trials now under way to directly compare the different AIs will hopefully provide clear results.

Androgens regulate growth of normal and neoplastic mammary cells in a cell type-specific manner, either by inhibiting or stimulating growth (44). However, the mechanisms by which androgens via AR regulate breast cancer growth remain elusive. Female AR knock-out mice exhibit decreased ductal branching and terminal end buds in prepubertal animals and retarded lobuloalveolar development in adult animals (45). Likewise, targeted disruption of AR in MCF-7 cells also leads to severe inhibition of proliferation (45). Epidemiologic analyses indicate a positive correlation between androgen levels and the incidence of breast cancer; meta-analysis from nine prospective studies showed that a doubling in testosterone concentrations in postmenopausal women translated into an increased relative risk of 1.42 unadjusted and 1.32 adjusted for E2 (46). AR status in breast cancer associates with both positive and negative indicators and clinical outcome. AR expression has been found in 84% (47) to 91% (48) of clinical breast cancers, and associated with ER status, but has also been found in 40% of ER-negative tumors (49). Patients with tumors that coexpress AR with ER and progesterone receptor have shown longer disease-free survival (DFS) than patients whose tumors were negative for all three receptors (48), but AR protein levels have also served as an independent predictor of axillary metastases in multivariate analysis (47) Furthermore, AR expression has correlated with decreased histopathologic grade, greater age, and postmenopausal status, but also lymph node–positive status (50). In AR-positive/ER-negative tumors, AR expression again associated with positive and negative indicators/outcome such as increased age, postmenopausal status, and longer DFS but also tumor grade, tumor size, and HER-2/neu overexpression (49).

Patients who fail AI therapy, whether the AI was steroidal or nonsteroidal, likely harbor tumor cells that have been selected for growth in an estrogen-depleted environment and, hence, are not dependent on ER activity for survival. Not all androgens are metabolized by aromatase to estrogens; for instance, dihydrotestosterone cannot be converted to an estrogen by aromatase (44). Thus, a possible mechanism for failure of AI therapy in the clinic is androgen-stimulated breast cancer growth, a largely unrecognized alternative mechanism. We observed cellular proliferation of T47D cells in response to R1881 and 17-hydroexemestane, and these effects were blocked by bicalutamide. Therefore, T47D cells contain a functional AR signaling pathway that promoted growth in the absence of estrogen. Because functional AR signaling could be etiologically involved in a subpopulation of clinical breast cancers, those patients who have AR-positive tumors and achieve high circulating levels of 17-hydroexemestane, yet whose disease progresses while on exemestane therapy, may respond to AR-based therapy such as the antiandrogen bicalutamide.

Acknowledgments

We thank Dr. Alan E. Wakeling and Dr. Barrington J.A. Furr for providing fulvestrant and bicalutamide, respectively. We also thank members of the Jordan laboratory for helpful discussions, and Dr. Jennifer L. Ariazi (GlaxoSmithKline, Collegeville, PA) for critical review of the manuscript.

References


Molecular Cancer Therapeutics

Exemestane's 17-hydroxylated metabolite exerts biological effects as an androgen

Eric A. Ariazi, Andrei Leitão, Tudor I. Oprea, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-07-0312

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2007/12/14/1535-7163.MCT-07-0312.DC1

Cited articles
This article cites 49 articles, 15 of which you can access for free at:
http://mct.aacrjournals.org/content/6/11/2817.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
/content/6/11/2817.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.