Aromatase Destabilizer: Novel Action of Exemestane, a Food and Drug Administration–Approved Aromatase Inhibitor

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Abstract

Using Western blot as the major technique, we studied the effects of the three Food and Drug Administration (FDA)–approved aromatase inhibitors (AI) on aromatase protein stability in the aromatase-overexpressing breast cancer cell line MCF-7aro. We have found that exemestane treatment significantly reduces aromatase protein level. Exemestane induces aromatase degradation in a dose-responsive manner (25-200 nmol/L), and the effect can be seen in as early as 2 hours. Metabolic labeling with 35S-methionine was used to determine the half-life (t1/2) of aromatase protein. In the presence of 200 nmol/L exemestane, the t1/2 of aromatase was reduced to 12.5 hours from 28.2 hours in the untreated cells. Furthermore, exemestane-induced aromatase degradation can be completely blocked by 10 μmol/L MG132, indicating that the degradation is mediated by proteasome. We also examined the effect of exemestane on aromatase mRNA level using real-time reverse transcription-PCR. No significant changes in mRNA level were detected after 8 hours of treatment with exemestane (200 nmol/L). This is the first report on the evaluation of three FDA-approved AIs on the stability of the aromatase protein. We have found that exemestane, different from letrozole and anastrozole, can destabilize the aromatase protein. (Cancer Res 2006; 66(21): 10281-6)

Introduction

Aromatase, a cytochrome P450 enzyme, catalyzes three consecutive hydroxylation reactions, converting C19 androgens to aromatic C18 estrogenic steroids. On receiving electrons from NADPH-cytochrome P450 reductase, aromatase converts androstenedione and testosterone to estrone and estradiol, respectively. As the key enzyme in estrogen synthesis, aromatase plays a crucial role in breast cancer development. Increasing evidence has shown that aromatase inhibitors (AI) are superior to the conventional anti-estrogen tamoxifen in treating hormone-dependent breast cancer in postmenopausal women (1–4). In the past three decades, a series of AIs have been produced. Historically, AIs have been grouped into three generations. As the prototype of ‘first-generation inhibitor’ aminoglutethimide was the first drug to be used as an AI (5). Its nonspecific inhibition on P450 enzymes, other than aromatase, caused significant side effects. The representative of ‘second-generation inhibitor’, 4-hydroxy-4-androstene-3,17-dione (4-OHA), was the first selective AI to be used clinically and was effective and well tolerated (6). Due to the extensive first-pass metabolism, 4-OHA needs to be administrated i.m. (7). The three FDA and Drug Administration (FDA)–approved AIs currently available in the United States (i.e., anastrozole, letrozole, and exemestane) are referred to as ‘third-generation inhibitors’. 4-OHA and current FDA-approved agents are all specific, more potent, and offer significant safety advantages over their nonselective predecessors (8). Based on their structures, AIs can be grouped into ‘nonsteroidal’ and ‘steroidal’ inhibitors. Nonsteroidal inhibitors (e.g., anastrozole and letrozole) have the triazole functional group that interact with the heme prosthetic group of aromatase and act as competitive inhibitors with respect to the androgen substrates. Steroidal inhibitors (e.g., exemestane and 4-OHA) were originally designed as substrate analogues that compete with the substrate for the aromatase enzyme. These two steroidal inhibitors are also mechanism-based inhibitors, which require the catalytic ability of active aromatase to convert them into active intermediates. The intermediates then bind irreversibly to the enzyme and cause its inactivation in a time-dependent manner. The development of AIs and their applications in breast cancer treatment have been reviewed recently by Brueggemeier et al. (9).

To facilitate the study of aromatase in breast cancer, our laboratory developed a breast cancer cell line MCF-7aro that stably expresses a high level of aromatase (10). In our effort to generate and characterize AI-resistant cells, we have noticed the decreased level of aromatase protein in exemestane-treated cells. This observation prompted us to look into the effects of three FDA-approved AIs in aromatase protein stability. With the well-established Western blot and immunoprecipitation procedures in our laboratory, we have examined exemestane-induced degradation of aromatase protein in MCF-7aro. We also used 35S-methionine metabolic labeling to determine the half-life (t1/2) of aromatase in the presence of exemestane. Furthermore, our experiments suggest that proteasome is a potential mediator of exemestane-induced aromatase degradation.

Materials and Methods

Cell culture. MCF-7aro cells were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS), 110 mg/L sodium pyruvate, 100 μmol/L nonessential amino acids, and 100 mg/L G418 (Invitrogen, Carlsbad, CA). Letrozole, anastrozole, and exemestane were provided by Novartis AG (Basel, Switzerland), Zeneca Pharmaceuticals (Macclesfield, United Kingdom), and Pharmacia Italia S.p.A. (Nerviano, Italy), respectively. Proteasome inhibitor MG132 and protein synthesis inhibitor cycloheximide were purchased from BioMol (Plymouth Meeting, PA). 4-OHA was purchased from Sigma-Aldrich (St. Louis, MO).

Western blot. Cells were cultured in 60-mm dishes and lysed with Celllytic M (Sigma-Aldrich) supplemented with protease inhibitor tablets (Roche, Indianapolis, IN). Protein lysate (60 μg) was loaded and separated on 10% SDS-PAGE gel. Protein transfer was done using Trans-Blot SD semidyry system (Bio-Rad, Hercules, CA) at 20 volt for 1 hour (transfer buffer: 25 mmol/L Tris, 192 mmol/L glycine, 20% methanol). For samples to be detected with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL), polyvinylidene difluoride membrane (Bio-Rad) was

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used and blocked with StartingBlock blocking buffer with Tween 20 (Pierce) at room temperature for 1 hour. For samples to be detected with the LI-COR Odyssey Infrared Imaging System (Lincoln, NE), nitrocellulose membrane was used and blocked with blocking buffer from LI-COR at room temperature for 1 hour. Rabbit anti-aromatase (Hauptman-Woodward Institute, Buffalo, NY) was diluted (1:1,000) and mouse anti-aromatase (Serotec, Raleigh, NC) was diluted (1:300). The specificity of both anti-aromatase antibodies has been shown previously (11, 12). Using appropriate negative (i.e., MCF-7 cells) and positive (i.e., recombinant aromatase and human placenta microsome preparation) controls, we have also confirmed the specificity of these antibodies in our laboratory. Membranes were incubated with primary antibody at 4°C overnight.

**S<sup>35</sup>-methionine metabolic labeling.** Cells were cultured in 60-mm dishes and were 90% confluent at the time of experiment. To pulse the cells with S<sup>35</sup>-methionine, normal culture medium was removed and 2 mL methionine-free DMEM, supplemented with 10% dialyzed FBS (Invitrogen), was added into each dish followed by addition of 12 µL NEG-772 EASYTAG Express protein labeling mix (2 mCi, 180 µL; Perkin-Elmer, Boston, MA). After 80 minutes of pulsing, S<sup>35</sup>-containing medium was removed and normal culture medium was added back, with or without 200 nmol/L exemestane. Cells were then lysed with 1 mL lysis buffer (the same buffer as used in Western blot) at 0, 2, 4, and 8 hours time points and stored at −80°C. After a complete set of samples was collected, lysates were spun at 12,000 x g for 12 minutes at 4°C and clear supernatant was collected. For immunoprecipitation, 5 µL rabbit anti-aromatase antibody (Hauptman-Woodward Institute) and 30 µL protein A/G Plus-agarose (Santa Cruz Biotechnology) were added into each sample and rotated at 4°C overnight. After washing four times with cold PBS, samples were heated at 94°C for 5 minutes in loading buffer [50 mmol/L Tris (pH 6.8), 2% SDS, 10% glycerol, 100 mmol/L DTT] and ran with 8% SDS-PAGE gel. Gels were dried and exposed to X-ray film overnight followed by densitometry analysis. The results were then expressed as percentage of 0 hour control. Regression analysis was done with Microsoft Excel.

**Real-time quantitative reverse transcription-PCR.** Trizol reagent (Invitrogen) was used for total RNA isolation. SYBR Green Supermix and iScript cDNA Synthesis kit (Bio-Rad) were used for cDNA preparation. PCR primers for aromatase were as follows: 5’GATGATGTAATCGATGGCTAC3’ and 5’TTCATCATCACATGGCGAT3’; for human β-actin, 5’AGAAAGAGATCACTGCCCCTGGCACC3’ and 5’CCTGCTTGCTGATCCACATCTGCTG3’. Annealing temperature for PCR was 58°C. Results were analyzed with the software provided with the iCycler iQ5 Real-time PCR Detection System (Bio-Rad).

**Aromatase assay.** “In-cell” aromatase assay was done according Zhou et al. (10) with modifications. Briefly, cells were seeded into 96-well plates.
and incubated with 100 nmol/L [1-β-3H]-4-androstene-3,17-dione in serum-free medium for various times. The reaction mixture was then extracted with dextran-coated charcoal, and the product, tritiated water, was counted with a liquid scintillation counter. Aromatase activity was expressed as pmol of tritiated water released per mg protein per hour (pmol/mg/h). For dose-responsive experiments, exemestane at various concentrations were included during the assay. For time-dependent aromatase inhibition by exemestane, cells were preincubated with 50 nmol/L exemestane for designated times. Exemestane-containing medium was then removed and the cells were washed twice with PBS and assayed for aromatase activity.

Results

Aromatase inhibitors affect the stability of aromatase protein. The two nonsteroidal AIs, letrozole and anastrozole, at concentration as low as 8 nmol/L, caused an increase of aromatase protein levels. These results were expected because letrozole and anastrozole bind to aromatase with high affinities that stabilize the structure of aromatase protein. However, it was not expected that the steroidal inhibitor, exemestane, in contrast, caused a reduction of the aromatase protein (Fig. 1A). To better understand the mechanism of the exemestane-induced aromatase degradation, the following experiments were done. We found that exemestane in the range of 25 to 200 nmol/L caused aromatase degradation in a dose-responsive manner. With 200 nmol/L exemestane, the degradation could be detected as early as 2 hours (Fig. 1B). Because exemestane is an androgen analogue, we questioned if androgen also had a similar effect on aromatase. Our Western blot showed that testosterone (range, 1-1,000 nmol/L) had no effect on the stability of aromatase protein (Fig. 1C) and further indicated that exemestane-induced aromatase degradation is the unique action of exemestane.

Exemestane destabilizes aromatase. Metabolic labeling using S[35]methionine is a well-established method to determine protein t1/2. MCF-7aro cells were pulsed with labeling mix for 80 minutes followed by incubation in DMSO vehicle control or 200 nmol/L exemestane. Samples were collected at 0, 2, 4, and 8 hours. Aromatase was immunoprecipitated with rabbit anti-aromatase antibody and run on 8% SDS-PAGE gel. After being exposed to X-ray film, densitometry analysis was done. In the control setting, aromatase had a t1/2 of ~28.2 hours. With the presence of 200 nmol/L exemestane, aromatase t1/2 was reduced to ~12.5 hours (Fig. 2).

Aromatase degradation is mediated by proteasome. To examine if the decrease in aromatase protein is caused by changes in the rate of de novo synthesis, we used an inhibitor of protein synthesis, cycloheximide. Our results showed that, under cycloheximide (20 ng/mL) plus exemestane, the overall aromatase signal was weaker than that in cells treated with exemestane only due to the inhibition on overall protein synthesis by cycloheximide (Fig. 3A). Under cycloheximide treatment, any change in aromatase protein can be attributed to degradation. It is clear that the 20 hours of incubation time with cycloheximide in this experiment is too long and caused significant protein loss, so we shortened the incubation to 8 hours in the following experiments. To see if exemestane alone can degrade aromatase, we incubated 1 µmol/L exemestane or DMSO control with purified aromatase in a test tube for 8 hours at 37°C followed by Western blot. No significant change of aromatase protein was detected (data not shown). Next, we used MG132, a specific proteasome inhibitor, to examine the role of proteasome in exemestane-induced aromatase degradation. With 10 µmol/L MG132, the exemestane-induced degradation was totally blocked (Fig. 3B and C). Our results clearly indicate the involvement of proteasome in aromatase protein degradation.

Aromatase inhibitors have no effect on aromatase mRNA. The ability of MG132 to block exemestane-induced aromatase degradation is a good indication that this process is regulated by proteasome at the protein level. However, it is still important to examine the effects of AIs on aromatase mRNA stability. Cells were treated with either 20 nmol/L letrozole, 20 nmol/L anastrozole, or 200 nmol/L exemestane for 8 hours. Then, total RNA was collected and quantitative real-time PCR was done. No significant differences were detected on mRNA level among the treatments (Fig. 3D). Clear differences on aromatase protein were detected under similar conditions. Clearly, the exemestane-induced aromatase degradation is not mediated at the mRNA level.

Exemestane inhibits aromatase in a dose-responsive and time-dependent manner. To confirm the inhibitory effect of exemestane in MCF-7aro cells, we did the "in-cell" aromatase assay. We show that exemestane inhibits aromatase dose responsively, with IC50 of ~20 nmol/L (Fig. 4A). Next, we verified the 'time-dependent' inhibition of aromatase by exemestane (Fig. 4B).
Exemestane is known as an irreversible, mechanism-base AI. This compound causes a time-dependent inactivation of human placental aromatase with a \( t_{1/2} \) of 13.9 minutes and \( K_i \) of 26 nmol/L (13). As shown in Fig. 4A, exemestane inhibited aromatase in MCF-7aro cells in a time-dependent manner. Based on previous knowledge and our current findings, as shown in Scheme 1, the interaction between exemestane and aromatase can be divided into three steps. Step 1 is the reversible step when exemestane binds to aromatase with a \( K_i \) of 26 nmol/L. At step 2, exemestane is converted into an intermediate, through a yet unknown process, and results in an irreversible inactivation of the enzyme. The \( t_{1/2} \) of this process is 13.9 minutes. At step 3, a degradation of aromatase by proteasome occurs after the irreversible inactivation step. We have found that in vitro incubation of exemestane with purified aromatase had no effect on aromatase protein stability. Following the exemestane treatment of MCF-7aro cells, the \( t_{1/2} \) of aromatase protein is reduced by 50% or more. We have determined that exemestane, at 200 nmol/L, decreases aromatase \( t_{1/2} \) to 12.5 hours from 28.2 hours in untreated cells. In addition, exemestane treatment did not cause significant changes of aromatase mRNA levels. Therefore, exemestane is a unique AI that can also destabilize aromatase protein.

There were a few reports on the effect of some earlier generations of AIs on aromatase stability. Foidart et al. (14) used an immunocytochemical procedure to study the immunoreactive aromatase in quail brain. They reported that two nonsteroidal inhibitors, fadrozole and vorozole, increased immunoreactive aromatase; whereas two steroidal inhibitors, 4-OH-androstenedione and androstatrienedione, decreased that signal. This report provided only limited insight on the effect of AI on aromatase stability due to the complexity of the in vivo system. Yue and Brodie (15) reported the possible role of 4-OHA in inducing aromatase degradation. In that study, they measured aromatase activity in human choriocarcinoma-derived JEG-3 cells, after treating the cells with cycloheximide or cycloheximide plus 4-OHA for different times. They found that aromatase activity in 4-OHA plus cycloheximide–treated cells were declining faster than that with cycloheximide alone. Because cycloheximide has no inhibitory effect on aromatase, the results suggest that 4-OHA induces aromatase degradation. Using an ELISA method, Harada et al. (16) studied the effects of 4-OHA on aromatase protein and mRNA levels.

**Figure 3.** Exemestane-induced aromatase degradation is mediated by proteasome. A, cells were treated with either exemestane (Exe) or exemestane with 20 ng/mL cycloheximide (CHX) for 20 hours. Wedge, decreasing concentrations of exemestane. Western blot was detected with enhanced chemiluminescence exposed to an X-ray film. B, cells were treated for 8 hours with either 1 μmol/L exemestane, 20 ng/mL cycloheximide, 10 μmol/L MG132, or their combinations. The Western blot was detected with Odyssey Infrared Imaging System. C, quantification of (B) using the software that provided with the Odyssey System. Aromatase band intensity was normalized over actin and expressed as average with SDs. D, cells were treated with 20 nmol/L letrozole, 20 nmol/L anastrozole, or 200 nmol/L exemestane for 8 hours. Total RNA was prepared and quantitative reverse transcription-PCR was done. Results are expressed as the ratio of aromatase over actin starting quantity. Numbers below the aromatase Western blot are the ratio of band intensity to the DMSO control. Each treatment has been done in triplicate. Representative of three experiments.
of several AIs on aromatase expression and protein levels in JEG-3 cells. They reported a time-dependent increase of aromatase protein with three nonsteroidal inhibitors (fadrozole, vorozole, and pentrozole) and a steroidal inhibitor (atametsane), whereas aromatase mRNA levels were not affected. Harada et al. (16) did not find any inhibitors that could induce aromatase degradation.

As the first AI approved in Europe and Canada, 4-OHA is structurally very similar to exemestane and is also a mechanism-based AI. For that reason, we also examined 4-OHA for its effect on aromatase protein. Our results show that 4-OHA can also induce aromatase degradation (Fig. 5). In addition to exemestane and 4-OHA, several additional mechanism-based AIs have been synthesized. These inhibitors have been discussed recently in a review by Brueggemeier et al. (9). Briefly, the first mechanism-based inhibitor was 10-propargyl-4-estrene-3,17-dione (MDL 18,962; refs. 17–19). The other two extensively studied mechanism-based inhibitors are 7α-(4-aminophenyl)-thioandrosta-1,4-diene-3,17-dione (7α-APTADD) and 7α-phenethyl-androsta-1,4-diene-3,17-dione (7α-PEADD; ref. 20). The exact nature of the interaction of these mechanism-based inhibitors with aromatase protein and amino acids involved are yet to be elucidated. Although we do not yet know whether MDL18,962, 7α-APTADD, and 7α-PEADD can induce aromatase degradation, the ability of exemestane and 4-OHA to induce enzyme degradation could explain why it has been difficult for the identification of amino acids/peptides participated in the mechanism-based inhibition of aromatase.

In conclusion, our present study provides direct evidences that among three third-generation AIs, exemestane is different from letrozole and anastrozole that it can also destabilize the target, aromatase protein, and point out the role of proteasome in mediating this degradation. Our finding will have an important effect in the use of exemestane in breast cancer treatment. In addition, previous studies from this and other laboratories have mainly focused on the transcriptional regulation of aromatase expression (21, 22). This study will draw attention to the area of protein stability as an important aspect of aromatase regulation. Knowledge gained from these studies should be helpful in designing a new line of drugs to treat hormone-dependent breast cancer.

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References


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