Aromatase Inhibition and Inactivation

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Abstract
Aromatase is the key enzyme in the synthesis of estrogens and mediates the conversion of androstenedione and testosterone to estrone and estradiol. Because of the importance of estrogen in stimulating breast cancers, the inhibition of estrogen synthesis is a logical approach to treatment. Aromatase is an excellent target for inhibition, because it is the last step in steroid biosynthesis, and, therefore, there are no important downstream enzymes to be affected. In addition, although aromatase is a P-450 enzyme and shares common features with other enzymes in this class, such as liver metabolizing enzymes and steroidogenic enzymes, it has unique features in the aromatizing reaction, features that are amenable to the development of selective inhibition. The approach we took to develop the first aromatase inhibitors was to design substrate analogues based on the structure of androstenedione. Some of these inhibitors, such as 4-hydroxyandrostenedione [4-OHA (later known as formestane)], also cause enzyme inactivation. Instead of being released at the end of the reaction, the substrate analogue remains bound. Therefore, the inhibitor is not required to be present at all times to maintain inhibition, and it has high enzyme specificity. Subsequently, other investigators have taken a different approach to developing compounds based on inhibitors of P-450 enzymes. High selectivity has been achieved with some of these reversible inhibitors. We have developed a unique animal model with human tumors to compare the antitumor efficacy of antiestrogens and aromatase inhibitors and to optimize their use in sequence and combination as a guide for future clinical trials.

Introduction
Aromatase is the key enzyme in the synthesis of estrogens and catalyzes the conversion of androstenedione and testosterone into estrone and estradiol. Estrogens are known to be important in the growth of breast cancers in both pre- and postmenopausal women. In premenopausal women, the ovarian granulosa cells are the major sites of estrogen synthesis. Aromatization also occurs in adipose tissue and muscle of both sexes. In postmenopausal women, extragonadal aromatization by these tissues is the main source contributing to circulating estrogen levels in postmenopausal women (1). However, breast tissue has been found to have several-fold higher levels of estrogen than those in the plasma of older women (2-4). Although in some patients a gradient favoring estrogen accumulation may account for higher tissue levels, a number of reports, including our own, indicate that aromatase activity as well as aromatase mRNA is present in normal breast tissue and breast tumors (5-11). Approximately 60% of breast tumors express aromatase (12) and have aromatase activity (13). These findings suggest local sources can contribute to relatively high concentrations of estrogen in the tumor environment.

Aromatase in extragonadal sites is not regulated by gonadotropins but by glucocorticoids, cAMP, prostaglandin E2, and other factors. Thus, in postmenopausal breast cancer patients, estrogen synthesis is independent of feedback regulation between the pituitary and the ovary. The tissue-specific manner of regulation of aromatase involves the use of alternative promoters (14). In the gonads, a promoter proximal to the translational start-site, promoter II, is used. In peripheral tissues, two promoters regulate the enzyme: promoter II and promoter I.4 (14, 15). In breast cancer, prostaglandin E2, the product of the inducible form of cyclooxygenase (COX-2), may be an important mediator of increased aromatase expression (12). Because of the importance of estrogen in stimulating breast cancer proliferation, the inhibition of estrogen synthesis is a logical approach to treatment. There is an increased incidence of cancer with age and also an increase in ER concentrations, making tumors of postmenopausal patients more sensitive to estrogens (16). Systemic treatment with aromatase inhibitors could be particularly useful for postmenopausal breast cancer patients.

Aromatase as Target for Inhibition
Aromatase is an excellent target for inhibition, because it mediates the last in the series of steps in steroid biosynthesis. Therefore, the inhibition of aromatase will not interfere with any downstream steroid biosynthesis. Although aromatase is a P-450 enzyme and shares common features with other enzymes in this class, the unique aromatization reaction, involving loss of the C-19 carbon and conversion of the steroidal-A-ring to an aromatic ring, provides the opportunity to develop inhibitors selective for P450arom (17).

The importance of selective inhibition is that potent inhibitors that bind to the target enzyme with high affinity are less likely to interact with other P450 enzymes, such as 11β-hydroxylase. The latter mediates the synthesis of the adrenal steroid cortisol and is inhibited along with aromatase and other P-450 enzymes by general inhibitors of steroid biosynthesis, such as aminogluthethimide. This compound was used in breast cancer treatment initially to produce “medical adrenalectomies” but later was used to inhibit aromatase in conjunction with cortisol replacement (18). Aminogluthethimide is a relatively weak inhibitor of aromatase but was useful in demonstrating that tumors respond to aromatase inhibition. Interaction with other enzymes

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and receptors at doses required to inhibit aromatase results in a number of toxicities. Selective inhibitors provide effective and better-tolerated drugs.

Selective Aromatase Inhibitors

The approach we took a number of years ago to develop the first aromatase inhibitors was to design substrate analogues based on the structure of androstenedione. In 1973, our laboratory demonstrated the first of a number of potent aromatase inhibitors (19). However, their use in the clinic did not begin until the early 1980s (20, 21) after preclinical development of 4-OHA,3 later known as formestane (22–25).

Enzyme Inactivators. Initially, the compounds we evaluated appeared to be typical competitive inhibitors. On subsequent analysis, it became evident that some of these inhibitors with steroidal structures, such as 4-OHA, also cause enzyme inactivation (26, 27). In vitro, 4-OHA interacts with human placental aromatase with an apparent \( K_i \) of 10.2 nm, causing rapid irreversible inactivation with a \( K_{i_{\text{irr}}} \) of 0.41 \( \times 10^{-3} \) s (26, 27).

These types of substrate analogues are thought to interact with the steroid binding region of the enzyme and are converted by the normal catalytic mechanism to a product that binds either very tightly or irreversibly by covalent binding to the enzyme, causing its inactivation (Fig. 1). Thus, the synthesis of estrogen is unable to occur until new enzyme is produced. The significance of the inactivation mechanism is that inhibitors of this type are highly specific for the enzyme. Only compounds that provide a close fit comparable with that of the natural substrate and can interact with the enzyme's catalytic mechanism will cause enzyme inactivation (28). In addition, once the enzyme is inactivated, no estrogen is produced until new enzyme is made. Therefore, it is unnecessary to have the drug present at all times to maintain inhibition of the enzyme, as is required with reversible inhibitors. High specificity and enzyme inactivation are properties that will result in drugs that are well tolerated. A number of other steroidal derivatives of the substrate androstenedione (26, 29–31) cause similar inactivation of aromatase to 4-OHA.

Promising preclinical results with 4-OHA (23) and initial clinical studies (20, 21) provided the basis for additional clinical trials of formestane. Formestane (4-OHA) was the first selective aromatase inhibitor to become available and the first new endocrine treatment for breast cancer in 10 years at that time. It is now approved in many countries worldwide for treatment of advanced breast cancer in postmenopausal women. Exemestane, an aromatase inhibitor also thought to cause enzyme inactivation, has recently been approved in the United States.

Nonsteroidal Aromatase Inhibitors. Subsequently, other investigators took a different approach to developing aromatase inhibitors. Some compounds were based on inhibiting P-450 enzymes and were derived from drugs used as antifungal agents such as ketoconazole, which inhibits fungal P-450 enzymes. Nonsteroidal inhibitors possess a heteroatom such as a nitrogen-containing heterocyclic moiety. This interferes with steroid hydroxylation by binding with the heme iron of cytochrome P-450. These compounds are reversible inhibitors of aromatase. Most nonsteroidal inhibitors are intrinsically less enzyme specific and will inhibit, to varying degrees, other cytochrome P-450-mediated hydroxylations in steroidogenesis (Fig. 2). The challenge in developing these types of inhibitors was to improve selectivity for aromatase so as not to interfere with other P-450 enzymes. For example, inhibition of 11-hydroxylase, which mediates cortisol synthesis, and of 18-hydroxylase, which produces aldosterone, would not be desirable.

Because many of the nonsteroidal compounds that have been evaluated as inhibitors of aromatase are not potent inhibitors of the enzyme, it seems likely that the nature of the nonheterocyclic moiety is important. This portion of the molecule may interact with aromatase via hydrogen and/or van der Waals bonding. The degree of compatibility or synergism between binding to the heme-iron and interaction with the protein residue may also be crucial.

Several triazole compounds noted for good pharmacokinetic properties have been investigated as aromatase inhibitors. Two compounds, anastrozole and letrozole, are highly selective for aromatase and were recently approved by the Food and Drug Administration (FDA) for first-line therapy in postmenopausal women with advanced breast cancer.

Intratumoral Aromatase Model as a Guide for Future Trials

Unlike most antiestrogens, aromatase inhibitors are not estrogenic and were, therefore, predicted to be more effective than antiestrogens in inhibiting tumor proliferation. Also, because of differences in mechanism and structure, antiestrogens and aromatase inhibitors would not be expected to be cross-resistant. To evaluate the different aromatase inhibitors and to compare them with antiestrogens, we have developed a unique animal model with human tumors. Using this model, we have investigated the use of these agents in sequence and in combination as a guide for future clinical trials.

Most studies on aromatase inhibitors were carried out

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3 The abbreviations used are: 4-OHA, 4-hydroxyandrostenedione; ER, estrogen receptor.
Initially in patients with advanced disease who had previously received, and had usually progressed, on first-line endocrine therapy. The low response rates to aromatase inhibitors seen in these patients does not reflect the marked reduction in serum estrogen levels that occur during aromatase inhibitor treatment. This lack of correlation reflects the emergence of estrogen-independent tumor growth. The use of inhibitors in first-line therapy for metastatic disease or in early-stage disease is more likely to indicate their antitumor activity. Other problems in human studies are the significant differences in trial design and patient population. For example, not all patients included in studies were known to be ER-positive. Also, clinical resistance to first- and second-line therapy was not consistently reported. Therefore, to provide information that would predict the effects of these agents in the clinic and as a guide to developing new protocols, we established an intratumoral aromatase model in nude mice to simulate the postmenopausal ER-positive breast cancer patient (32, 33). The model is useful for comparing aromatase inhibitors and antiestrogens because the tumors are ER-positive.

The Xenograft Model. Because the rodent has no significant production of estrogen from nonovarian tissue, we used estrogen-dependent human breast cancer cells (MCF-7) transfected with the human aromatase gene (MCF-7CA) as an endogenous source of estrogen to stimulate tumor formation in ovariectomized nude mice (32, 33).

MCF-7 cells transfected with the human aromatase gene (MCF-7CA; 3 \times 10^7/ml cells in Matrigel) are inoculated into four sites in ovariectomized female BALB/c mice (ages 4–6 weeks). Throughout the experiment, animals receive s.c. injections of 0.1 mg/mouse/day androstenedione, the substrate for aromatization to estrogens. Tumor growth is measured with calipers weekly, and tumor volumes are calculated. When all tumors reach a measurable size (~500 mm³), usually 28–35 days after androstenedione injections are started, animals are assigned to groups of four or five mice and treatment is begun.

At necropsy, 4–6 h after the last injected dose, tumors are removed, cleaned, and weighed.

Comparison of Aromatase Inhibitors and Antiestrogens In the Mouse Model. The intratumoral aromatase model was used to investigate the effects of the nonsteroidal aromatase inhibitors letrozole and anastrozole and to compare them with the antiestrogens tamoxifen and fulvestrant (Faslodex). These studies simulate first-line therapy. Because the tumors synthesize and respond to estrogen, antiestrogens and aromatase inhibitors can be directly compared. We found that, although the antiestrogens tamoxifen and fulvestrant and the aromatase inhibitors letrozole and anastrozole were all effective in reducing tumor growth, both of the aromatase inhibitors were more effective than tamoxifen (34, 35), as subsequently observed in the clinical trials. Anastrozole (Arimidex; 5 μg/day), in contrast with tamoxifen (3 μg/day), caused significant inhibition of tumor growth compared with the controls in the tumor model (P < 0.05; Refs. 34, 35). Letrozole (Femara; 10 μg/day) was the most potent compound and was more effective than tamoxifen (60 μg/day) and fulvestrant (ICI 182,780; 5 mg/week), although both fulvestrant and letrozole showed reversion of established tumors. Letrozole (5 μg/day) was also able to cause marked regression of large tumors (36). Treatment with letrozole (5 μg/day) resulted in regression of tumor growth for up to ~15 weeks with continuous treatment. Thereafter, the tumors gradually resumed growth and almost reached their initial volume by 19 weeks of treatment (35).

The Effect of Combining Aromatase Inhibitors with Antiestrogens. Because both antiestrogens and aromatase inhibitors are effective in treating breast cancer patients, combining these agents with different mechanisms of action might result in greater antitumor efficacy than either alone. A clinical trial [ATAC (Anastrozole Tamoxifen and Combination) trial] is currently in progress to investigate this possibility using anastrozole and tamoxifen. We have used the intratumoral aromatase model to determine the efficacy of this strategy. In these experiments, we used low doses of the compounds, which cause partial tumor suppression, to determine whether the compounds have synergistic or additive effects and would result in greater reduction in tumor growth. Because previous studies of 10 μg/mouse/day letrozole caused almost complete regression of tumors, a dose of 5 μg/day letrozole or anastrozole was used in the combined treatments with antiestrogens. The dose of tamoxifen was 3 μg/day. Fulvestrant, a pure antiestrogen, (5 mg injected in oil once weekly) was also compared. All of the compounds alone, or in combination, at these doses were effective in suppressing tumor growth in comparison with the control mice. Weights of tumors removed at the end of treatment were reduced significantly more than with tamoxifen (P < 0.05; Refs. 34, 35). Letrozole (Femara; 10 μg/day) was the most potent compound and was more effective than tamoxifen (60 μg/day) and fulvestrant (ICI 182,780; 5 mg/week), although both fulvestrant and letrozole showed reversion of established tumors. Letrozole (5 μg/day) was also able to cause marked regression of large tumors (36). Treatment with letrozole (5 μg/day) resulted in regression of tumor growth for up to ~15 weeks with continuous treatment. Thereafter, the tumors gradually resumed growth and almost reached their initial volume by 19 weeks of treatment (35).

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The effect of combining aromatase inhibitors and antiestrogens on growth of MCF-7CA breast tumors in the nude mouse model. Groups of mice with four tumors each were treated with tamoxifen (3 μg/mouse/day), fulvestrant (ICI 182,780; 70 μg/mouse/week), anastrozole (ZD 1033; 5 μg/mouse/day), letrozole (CGS 20,267; 5 μg/mouse/day), and the combination of aromatase inhibitors and antiestrogens. Tumor volumes were measured weekly (from Ref. 36).

Time-to-Treatment Failure of Tamoxifen and Letrozole. In the more recent studies, we have treated mice with tumors of MCF-7CA cells with a much higher dose of tamoxifen (100 μg/mouse/day) than used above and 10-fold higher than the concentration of the aromatase inhibitor (letrozole 10 μg/mouse/day; Ref. 12). In addition, treatment was extended to determine the time-to-treatment failure. As shown in Fig. 4 and consistent with previous findings (34, 36), treatment with letrozole and with the combination of letrozole and tamoxifen caused tumor regression. Also, both treatments were more effective than tamoxifen alone. However, the combination was not more effective than letrozole alone. Reduction in tumor growth but not tumor regression was seen with tamoxifen alone. In addition, the time-to-treatment failure with tamoxifen was 8 weeks. Therefore, tumors began to grow despite continued tamoxifen treatment, whereas growth suppression was maintained with letrozole. Similar findings have recently been reported for first-line treatment of patients who relapsed sooner on tamoxifen than on letrozole. When mice were killed at 16 weeks, there was a marked reduction in tumor weights (P < 0.01) in the two letrozole-treated groups compared with mice treated with tamoxifen only.

In conclusion, aromatase inhibitors may offer better control of tumor growth than does the antiestrogen tamoxifen, not only by a greater effect on tumor volume and weight but also by extending treatment for a significantly longer period than with tamoxifen treatment alone. Combining the two types of compounds, aromatase inhibitors and antiestrogens, does not appear to result in better suppression of tumor growth than does using the aromatase inhibitors.

Open Discussion

Dr. James Ingle: Could you tell me what is known about de novo aromatase synthesis? Let’s say you block synthesis with a steroidal aromatase inhibitor. How long does it take to get the aromatase back?

Dr. Angela Brodie: We’ve measured in animal models and in cell cultures, where it seems to take about 24 h to come back up to initial levels.

Dr. Anthony Howell: The molecular studies show that the triazoles bind in the pocket similar to the steroidal compounds, and I wondered why we seem to have non-cross-resistance between those two groups of compounds.
Fig. 4  The time-to-treatment failure of letrozole and tamoxifen on growth of MCF-7C_A breast tumors in nude mouse model. Groups of mice with four tumors each of MCF-7C_A cells were treated as follows for 16 weeks: letrozole (10 μg/day; n = 6); tamoxifen (100 μg/day; n = 5); a combination of both compounds (n = 6). Tumor volumes were measured weekly. LET, Letrozole; TAM, Tamoxifen.

Dr. Brodie: They’re entirely different structures, and they’re binding to different parts of the enzyme. I think it’s largely because they’re completely different chemical structures that they don’t have cross-resistance.

Dr. Per Lonning: One controversial issue is whether you can actually bind a type II or type I inhibitor at the same time to the same enzyme, because it has been argued that if you bind to one binding site, it will preclude the binding to other binding sites because of three-dimensional configuration. Will you comment on that?

Dr. Brodie: I think that may well occur and that there will be no advantage. We haven’t actually done the experiments because we thought we probably wouldn’t get any additional inhibition by binding the two types of compounds because of just what you said. But it probably would be worth doing the experiments because a number of people have actually raised this question.

Dr. Matthew Ellis: Surely you can just bind an androgen to the enzyme and see whether it’s displaced by letrozole. So the experiments are very simple. The reason I think it is important is Dick Santen’s work, and now we’re learning about a mutant estrogen receptor that is supersensitive to picomolar concentrations of estrogen. It may in fact turn out to be important to suppress estrogen to levels we never dreamed were important. In the metastatic patient, does tumor regrowth happen because the tumor has educated itself to live off the meager pickings left by the inhibitor, or has it become estrogen independent? Probably both mechanisms are happening, but in different patients.

Dr. Brodie: Actually, in both the studies Santen has done and that we’ve done ourselves, the cell does adapt to living in very low concentrations of estrogen. The estrogen receptor level actually increases so that the cell becomes more and more sensitive. Certainly, this mutant could play a role as well. But thus far letrozole has been extremely effective in getting rid of the estrogen produced in the animal model.

Dr. Steven Come: What do we know about resistance to these compounds? Obviously, some tumors may just grow out so they’re no longer estrogen sensitive at all, but what about the ones that clinically respond to other hormonal agents?

Dr. Brodie: We have done studies in our mouse model looking at that, and clearly they remain sensitive. We’ve grown tumors out on letrozole, which takes a very long time, and then they remain sensitive to antiestrogens. They’re somewhat sensitive to tamoxifen, but they’re very sensitive to Faslodex. Part of that response may be because the estrogen receptor level is increased when they’re grown on letrozole and deprived of estrogen for a very long time.

Dr. Lonning: Together with Tony Howell, we have just completed a trial in which we took patients who had become resistant to treatment with aromatase inhibitors and gave them high-dose estrogens. A lot of them responded, so clearly they hadn’t lost the sensitivity to high-dose estrogen therapy. That was probably related to the bell-shaped curve for estrogen stimulation in vitro, but we don’t fully know yet.

Dr. Kent Osborne: You mentioned 99.99% elimination of estrogen, but that’s not really low when you’re thinking about the 10^{-15} M estrogen that probably stimulates some of these receptors. Even the wild-type receptor adapts, as you say, to very low estrogen levels. Maybe it up-regulates coactivators in addition to the receptor itself. High-dose estrogen, I think, is a valuable therapy, maybe particularly after the patient has adapted to very low estrogen levels.

Dr. Lonning: I want to go back to the mechanism of high-dose estrogen. So is the idea here that by giving a huge dose of estrogen to these hormone receptors, you’re squelching transcription factors that are otherwise critical for other survival growth pathways?

Dr. Brodie: That’s the theory proposed at this point, yes. There are not a lot of data on these other pathways.

Dr. Lonning: In our high-dose estrogen study, the median number of previous endocrine therapies was four. Of 32 pa-
tients, 10 achieved an objective response, some with duration for more than 2 years. We know that there is a bell-shaped curve. You move the sensitivity window to the left and that can sensitize the patients to high-dose estrogens. It’s just consistent with the observations that we know.

**Dr. Brodie:** It was well-known for a long time that steroids have a dose-response curve, that they’re stimulatory at low doses and inhibitory at high doses.

**Dr. Ingle:** Do you have any data with steroidal aromatase inhibitors in your mouse model with the MCF-7 transfected cells?

**Dr. Brodie:** We do with formestane. As far as combining formestane with antiestrogens, the inhibitor alone was always better. There was no additional benefit to combination compared to sequential treatment, and that’s been demonstrated in the clinic.

**Dr. Ellis:** There’s a group in Germany looking at the letrozole data and relating responsiveness to body mass index. The idea is that if the patient is very overweight, they may require increasing doses to achieve adequate inhibition. I don’t know whether there were sufficient numbers in your study, Dr. Lonning, to see if there’s any relationship between inhibition of total body aromatase and the body mass index.

**Dr. Lonning:** No, we don’t have the patient numbers to answer that.

**Dr. Ellis:** Come to North Carolina.

**Dr. Lonning:** I was thinking that, but I was too polite to say it.

**Dr. Brodie:** Actually, there were some preliminary data that Mitch Dowsett and Charles Coombes had some years ago. I don’t know how many patients, but we noticed that there was less estrogen suppression in some women, and it did turn out that they were the obese patients.

**References**


