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Aromatase and its inhibitors ☆

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Abstract

Inhibitors of aromatase (estrogen synthetase) have been developed as treatment for postmenopausal breast cancer. Both steroidal substrate analogs, type I inhibitors, which inactivate the enzyme and non-steroidal competitive reversible, type II inhibitors, are now available. 4-hydroxyandrostenedione (4-OHA), the first selective aromatase inhibitor, has been shown to reduce serum estrogen concentrations and cause complete and partial responses in approximately 25% of patients with hormone responsive disease who have relapsed from previous endocrine treatment. Letrozole (CGS 20, 269) and anastrozole (ZN 1033) have been recently approved for treatment. Both suppress serum estrogen levels to the limit of assay detection. Letrozole has been shown to be significantly superior to megace in overall response rates and time to treatment failure, whereas anastrozole was found to improve survival in comparison to megace. Both were better tolerated than the latter. The potential of aromatase within the breast as a significant source of estrogen mediating tumor proliferation and which might determine the outcome of inhibitor treatment was explored. Using immunocytochemistry and in situ hybridization, aromatase and $mRNA_{arom}$ was detected mainly in the epithelial cells of the terminal ductal lobular units (TDLU) of the normal breast and also in breast tumor epithelial cells as well as some stromal cells. Increase in proliferation, measured by increased thymidine incorporation into DNA and by PCNA immunostaining in response to testosterone was observed in histocultures of breast cancer samples. This effect could be inhibited by 4-OHA and implies that intratumoral aromatase has functional significance. An intratumoral aromatase model in the ovariectomized nude mouse was developed which simulated the hormone responsive postmenopausal breast cancer patient. This model also allows evaluation of the efficacy of aromatase inhibitors and antiestrogens in tumors of estrogen receptor positive, human breast carcinoma cells transfected with the human aromatase gene. Thus, the cells synthesized estrogen which stimulated tumor formation. Both aromatase inhibitors and antiestrogens were effective in suppressing tumor growth in this model. However, letrozole was more effective than tamoxifen. When the aromatase inhibitors were combined with tamoxifen, tumor growth was suppressed to about the same extent as with the aromatase inhibitors alone. Thus, there was no additive or synergistic effects of combining tamoxifen with aromatase inhibitors. This suggests that sequential treatment with these agents is likely to be more beneficial to the patient in terms of longer response to treatment.

1. Introduction

As with most forms of cancer, the incidence of breast cancer increases with age. Thus, breast cancer is more common among postmenopausal women than younger women. Nevertheless, the sensitivity of breast cancer to estrogen has also been found to increase as patients age. Two thirds of breast cancers in postmenopausal women (compared with less than half of premenopausal women) have tumors that are positive for estrogen and progesterone receptors (ER, PR), are dependent on these hormones for their proliferation, and are more likely to be responsive to hormonal therapy [1].

Although the ovary is no longer producing estrogen and progesterone after menopause, estrogen synthesis

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increases in peripheral tissues. Therefore, in postmenopausal patients, total blockade of estrogen is more likely to be accomplished with systemic treatment rather than surgical removal of endocrine glands. Two pharmacologic approaches are currently used to reduce the effects of estrogen: (1) inhibition of estrogen *action* by antiestrogens, which interact with estrogen receptors in the tumor, and (2) inhibition of estrogen *production* by inhibitors of aromatase (estrogen synthetase).

The antiestrogen tamoxifen has proved to be a significant advance in the treatment of breast cancer [2]. Nevertheless, due to its partial agonist activity, long term treatment with tamoxifen has been found to cause endometrial proliferation which has resulted in endometrial cancer in some patients. In addition, resistance to tamoxifen inevitably develops in breast tumors and results in disease progression [3]. To address these problems, we proposed the second approach of reducing estrogen production as a therapeutic strategy. Starting in the early 1970 s, we identified a number of compounds that are selective inhibitors of estrogen synthetase (aromatase) [4,5]. We considered that the more complete estrogen blockade via aromatase inhibition might result in greater tumor response than with tamoxifen. Since inhibitors of aromatase act by a different mechanism of action than tamoxifen and do not have estrogenic activity, they are not associated with stimulatory effects on the endometrium. Furthermore, by reducing estrogen production, aromatase inhibitors can elicit responses in some patients who have relapsed on antiestrogen therapy. Thus, aromatase inhibitors can extend the duration of response and quality of life for breast cancer patients.

Aromatase is a cytochrome P-450 hemoprotein which catalyzes the conversion of androgens, androstenedione and testosterone via three hydroxylation steps to estrone and estradiol. The first two occur on the C-19 methyl group while the final hydroxylation step is still uncertain. These reactions are discussed in detail in several reviews [6,7]. Although aromatase has features in common with other steroidogenic P-450 enzymes, the heme binding region which has the greatest homology has only 17.9–23.5% amino acids identical to those of other steroidogenic P-450 enzymes [8]. This suggests that P-450$_{arom}$ belongs to a separate gene family which has been designated CYP19 [9].

Recognizing the unique features of the reaction catalyzed by P-450$_{arom}$, we reasoned that selective inhibition of P-450$_{arom}$ might be achieved with substrate analogues. Furthermore, aromatase is a good target for selective inhibition because estrogen production is the last step in the biosynthetic sequence of steroid synthesis. Selective blockade of P-450$_{arom}$ will not interfere with the production of other steroids, such as adrenal corticoids.

2. Aromatase inhibitors

Following our initial publication of aromatase inhibitors [4], we [5,10] and others have identified a number of steroidal [11,12] (type 1 inhibitors) and non-steroidal inhibitors (type II inhibitors) [13,14]. Several of these have proved to be effective in animal tumor models. We carried out extensive endocrine and antitumor studies with 4-hydroxyandrostenedione (4-OHA) [5,15–18] a selective steroidal substrate analog which causes inactivation of aromatase [19]. Clinical studies show that this compound reduces peripheral aromatization and plasma estrogen levels. In postmenopausal breast cancer patients, comparison between the 250 mg and 500 mg IM doses administered every 2 weeks showed similar degrees of estrogen suppression. The overall response rates observed in several studies of breast cancer patients with advanced disease were complete or partial tumor regression in 25–39% of patients, disease stabilization in 22% of patients [20–24], and disease progression in the remaining women. When 4-OHA was administered orally (250 mg/day) to patients, the response rates were similar to those of patients receiving biweekly injections of 250 or 500 mg [25]. Biweekly injections of both doses of 4-OHA IM were well tolerated, with mild side effects occurring in 17% of patients. Because the differences in estrogen reduction between the treatment groups were minor and there were no significant differences in clinical efficacy, 250 mg of 4-OHA injected every two weeks is recommended since this has better local tolerability than the 500 mg dose [22]. 4-hydroxyandrostenedione (4-OHA, formestane, Lentaron®), the first selective aromatase inhibitor to be used clinically [20,21], is now proving to be effective in tamoxifen-resistant breast cancer patients and is available in many countries world wide.

Recently, two non-steroidal inhibitors, anastrozole (ZN 1033) and letrozole (CGS 20,267), have been approved for treatment in postmenopausal patients with advanced breast cancer. Anastrozole and letrozole are triazole derivatives, and are competitive and reversible aromatase inhibitors which are highly potent and selective for aromatase. In two recent studies of anastrozole, oral doses of 1 and 10 mg daily were compared with megestrol acetate (Megace®) 40 mg 4 times daily in postmenopausal breast cancer patients with advanced disease [26,27]. Complete or partial responses lasting 3–8 months were similar with both doses of anastrozole and with Megace (10.3% of patients receiving 1 mg anastrozole, 8.9% of patients receiving 10 mg anastrozole, and 7.9% of patients receiving

megestrol acetate). The disease was stabilized in 25.1, 22.6, and 26.1% of patients, respectively. Responses were observed in patients who progressed after receiving adjuvant tamoxifen as well as in patients who received tamoxifen for advanced disease. Follow-up of the two studies combined at 31 months [28] found significant improvement in overall survival in the patients receiving 1 mg anastrozole compared with patients receiving megestrol acetate (hazard ratio 0.78, $P = 0.02$). The anastrozole 1 mg group had a longer median overall survival (26.7 months vs 22.5 months) and higher 2 year survival (56.1 vs 46.3%) than the megestrol acetate group. The studies individually were consistent, as each demonstrated a lower risk of death for patients treated with anastrozole 1 mg compared to megestrol acetate (hazard ratios 0.74, $P = 0.048$, and 0.85, $P = 0.34$). The comparison of the anastrozole 10 mg group to megestrol acetate also demonstrated improved survival (hazard ratio 0.83, $P = 0.10$). Response and time to progression were not significantly different between treatments. However, these data are clinically significant as the survival benefit seen in the anastrozole 1 mg patients adds to the tolerability benefits previously reported.

Recently, a phase I study was carried out with letrozole in 21 postmenopausal patients with advanced breast cancer [29,30]. These patients in three successive groups ($n = 7$ per group) received oral letrozole 0.1, 0.5, or 2.5 mg per day. It was observed that estradiol and estrone in all three groups were suppressed to about the same extent and reduced to undetectable levels in many of the patients, despite the use of a highly sensitive assay. In addition, 33% of patients responded to treatment, while 23.8% had stable disease for more than 3 months. Another study confirmed these results with objective responses which lasted from 40 to 63 weeks in 5 of 14 patients [31]. A dose response effect was observed in one study. Thus, objective responses occurred in 28% of patients receiving 0.5 mg per day and in 39% of those receiving 1 mg per day. Stable disease was seen in 41 and 40% of patients in the two groups, respectively; few adverse events were reported.

Letrozole has also been compared at 0.5 mg once daily and 2.5 mg once daily, with megestrol acetate 160 mg once daily in a total of 551 postmenopausal women with advanced breast cancer previously treated with antiestrogens. Letrozole 2.5 mg once daily was found to be statistically superior to megestrol acetate in overall tumor response rate and time to treatment failure. Megestrol acetate was associated with significantly more adverse experiences that resulted in withdrawal from treatment than either dose of letrozole. In addition, there was significantly greater benefit of the 2.5 mg dose compared to the 0.5 mg dose of letrozole in terms of objective response rates, time to progression, time to treatment failure, and survival.

3. Aromatase expression in the breast

As indicated above, following menopause estrogens are synthesized in extragonadal tissues. Since muscle and fat make up a large proportion of the body mass, aromatization of adrenal androgens in these tissues are the main source of circulating estrogens. While serum levels in postmenopausal women are typically very low, several studies have reported that concentrations of estrogen in breast tissue are 4–6 fold higher and similar to those in premenopausal patients [32]. Furthermore, estrogen concentrations in tumors are higher than in breast fat [33,34]. These findings suggest that estrogens produced within the breast may be critical in stimulating tumor proliferation. Thus, effective inhibition of breast aromatase could be an important determinant of the outcome of aromatase inhibitor treatment. These possibilities prompted us to investigate aromatase expression in the breast by immunocytochemistry using a specific monoclonal antibody [35]. Our studies showed that the site of aromatization is mainly in the tumor epithelial cells of human breast cancers and the epithelial cells of the TDLU in the normal breast, although some stromal cells surrounding the tumor also contained the enzyme [35]. In situ hybridization with sequence specific probes confirmed these results. Furthermore, aromatase activity measured in cryosections of tumors correlated with a marker of proliferation (proliferating cell nuclear antigen score), suggesting that locally produced estrogens may stimulate the growth of the tumor. We also found that proliferation of some tumors in histoculture was enhanced by testosterone as well as estrogens. Testosterone stimulation could be abrogated by aromatase inhibitors, suggesting that estrogens were produced by the tumors via aromatization of testosterone. These studies suggest that aromatase is localized to a specific area of breast tissue and that the tumor may produce sufficient estrogen to be a relevant source stimulating tumor proliferation.

4. Intratumoral aromatase model

The clinical studies of aromatase inhibitors to date have been important in demonstrating the benefits of these agents for treating breast cancer patients with advanced disease. However, while serum estrogen levels are markedly reduced in all patients treated with aromatase inhibitors, there is limited information about the extent of aromatase inhibition in the breast during treatment [36]. A further complication of the

current trials is that most breast cancer patients receive tamoxifen first and then later relapse before receiving aromatase inhibitor treatment. Thus, the low response rates compared to the marked reduction in serum estrogen levels during aromatase inhibitor treatment, are likely to be influenced by hormonal insensitivity of some tumors or to other forms of drug resistance that may develop. Therefore, the efficacy of aromatase inhibitors has been difficult to determine from the trials to date.

In order to investigate the importance of intratumoral aromatase and also to compare the antitumor activity of aromatase inhibitors and antiestrogens, we recently developed an intratumoral aromatase model in nude mice. This model simulates the postmenopausal breast cancer patient, as ovariectomized BALB/c athymic (nude) mice are inoculated with estrogen dependent human breast cancer cells (MCF-7) transfected with the human aromatase gene [37,38]. Since the rodent has no significant production of estrogen from non-ovarian tissue, the MCF-7 cells transfected with the aromatase gene (MCF-7_{CA}) provide the source of estrogen to stimulate tumor formation. Thus, tumors produced from these MCF-7_{CA} cells grow faster that those in the same animal without aromatase [37] or which depend on circulating estrogen from neighboring tumors [39]. Recently, we have used this intratumoral model to investigate the effects of letrozole and anastrozole on tumor growth, as well as on the uterus. We compared these effects with those of the antiestrogen tamoxifen alone and in combination with the aromatase inhibitors [40].

Following inoculation of MCF-7_{CA} cells into four sites in each mouse treatment began in about 20–30 days when tumors had reached a measurable size (≈ 1 cm^3). The mice were then assigned to groups of four or five and injected daily sc. The aromatase inhibitor, letrozole (CGS 20267) was kindly provided by Dr A. Bhatnagar, Novartis Pharma, Basel, Switzerland. The aromatase inhibitor, anastrozole (ZN 1033) and the antiestrogen, tamoxifen were gifts from Dr A. Wakeling, Zeneca, Macclesfield, UK. The mice were administered 3 or 60 μg/mouse/day tamoxifen, 5, 10 or 10 μg/mouse/day letrozole or anastrozole in 0.3% hydroxypropyl cellulose (HPC), or vehicle (0.3% HPC, 0.1 ml/mouse /day). In experiments using combined treatments, the doses administered were the same as above. After 4–5 weeks, mice were autopsied 4 or 6 h after the last injection. The uteri and multiple small tumors at each inoculation site were removed and weighed.

All tumors in the control mice continued to increase in volume throughout the course of the experiments. Treatment with both aromatase inhibitors, letrozole and anastrozole, as well as the antiestrogen, tamoxifen were effective in reducing tumor growth [40]. A dose response effect with tamoxifen was evident. Thus, tamoxifen treatment at 60 μg/day caused a greater inhibition of tumor growth than treatment with 3 μg/day. However, tamoxifen (60 μg/day) was less effective than the aromatase inhibitor, letrozole which showed marked tumor suppression at 10 μg/day and 60 μg/day in this experiment (Fig. 1) [41]. In a subsequent experiment, letrozole (5 μg/day) not only reduced tumor growth but also caused regression of tumors, a result not previously noted with endocrine treatments in nude mice. Thus, tumors of a group of mice autopsied before the start of treatment were significantly greater (53.5 ± 7.5 mg) than those removed from animals after treatment with letrozole (60 μg/mouse/day) (20.6 ± 2.1 mg) ($P < 0.05$) [40]. This finding has recently been confirmed in subsequent experiments and one not previously noted with endocrine treatments in nude mice.

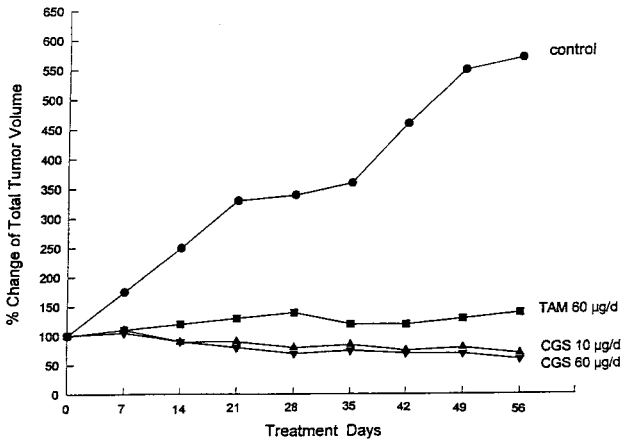

Fig. 1. Effect of tamoxifen and letrozole on tumor growth in the nude mouse. Ovariectomized nude mice were inoculated sc with MCF-7 cells stably transfected with the human placental aromatase gene (MCF-7_{CA}) in four sites and injected with 0.1 mg/mouse/day of androstenedione sc. Treatments began 21 to 35 days after inoculation of the cells, when the tumors had reached a measurable size. Letrozole (CGS), 10 and 60 μg/mouse/day, and tamoxifen (TAM), 60 μg/mouse/day, or vehicle (controls) in 0.3% hydroxypropyl cellulose (HPC) were injected sc for 56 days. Tumors were measured weekly, and the percentage change in total tumor volume is shown [41].

The MCF-7_{CA} tumors in the mice synthesize sufficient amounts of estrogen not only to support estrogen dependent tumor growth but also to maintain the uterus of these ovariectomized animals at a weight comparable to that of intact mice producing ovarian estrogen. Inhibition of intratumoral aromatase by letrozole treatment resulted in reduced estrogen production and a decrease in the mean uterine weight compared to that of the control mice ($P < 0.01$). There was no significant effect on the mean weight of uteri of the tamoxifen treated animals. As suggested in previous reports [42], tamoxifen is probably acting as an estrogen on the uterus.

Since both antiestrogens and aromatase inhibitors

Table 1
The effect of aromatase inhibitors and tamoxifen treatment on tumor weight in ovariectomized mice with MCF-7_{CA} tumors[a]

Treatment	Tumor weight (mg)
Vehicle	297.7 ± 54.5
Tamoxifen	178.9 ± 22.9
Letrozole	22.9 ± 3.7[b]
Anastrozole	104.3 ± 14.3[b]
Tam + Ana.	132.9 ± 26.5[b]
Tam + Let.	51.9 ± 8.9[b]

[a] Groups of four mice were treated with vehicle, letrozole (CGS20,267) (5 μg/day), anastrozole (ZD1033) (5 μg/day), tamoxifen (3 μg/day), and combinations at the same doses. Mice were autopsied after 42 days, tumors were removed and weighed. Values are mean ± SE. All values are significantly different from the controls ($P < 0.05$).
[b] Values are significantly different from tamoxifen ($P < 0.05$) [43].

are effective in breast cancer patients, combining treatments with these two mechanisms of action may provide greater efficacy than either alone. We have utilized the intratumoral aromatase model to address this question and to provide a guide to future clinical strategies. In order to determine whether greater reduction in tumor growth could be achieved by combining the two types of agents, we used low doses of the compounds which resulted in partial tumor suppression. As previously tested, 3 μg/day dose of tamoxifen and 5 μg/day dose of anastrozole and letrozole were used in the combined treatments. Tumor volumes, measured weekly over 42 days of treatment demonstrated that all compounds alone or in combination were effective in suppressing tumor growth in comparison to that of the control mice. Weights of tumors removed at the end of treatment were significantly reduced by treatment with letrozole and anastrozole compared to tamoxifen ($P < 0.05$) (Table 1). When combined with tamoxifen, the aromatase inhibitors did not produce greater reductions in tumor growth, as measured by tumor weight, than either letrozole or anastrazole treatment alone. There was a trend towards the combination of aromatase inhibitor and tamoxifen being less effective than the aromatase inhibitor alone but this was not statistically significant for either letrozole or anastrazole [43]. Similar results were also obtained when 4-OHA was combined with tamoxifen [38]. This suggests that tamoxifen might have partial agonist effects on the tumors which override the reduction in estrogen concentration produced by the aromatase inhibitors. Alternatively, interaction between the aromatase inhibitors and tamoxifen may alter the clearance of the aromatase inhibitor and result in less inhibition of estrogen synthesis. Nevertheless, it is evident that no additional benefit is likely to be gained by combining these two classes of agents for breast cancer treatment. Sequential use of aromatase inhibitors and antiestrogens is likely to provide a longer period of response for breast cancer patients taking these well tolerated drugs.

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