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Aromatase Inhibitors in the Treatment of Breast Cancer

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A number of inhibitors of estrogen synthesis are now becoming available which could be of value in the treatment of breast cancer. 4-Hydroxyandrostenedione (4-OHA), the first of these compounds to enter the clinic has been found to be effective in postmenopausal patients who have relapsed from tamoxifen. Thus, in studies of 240 patients, 26% patients experienced partial or complete response to treatment. An additional 25% patients had disease stabilization. 4-OHA is a potent selective, steroidal inhibitor which causes inactivation of aromatase *in vitro*. It is effective in reducing concentrations of ovarian estrogens in rats and of ovarian and peripheral estrogens in non-human primate species. The compound has been shown to lower serum estrogen levels in postmenopausal breast cancer patients. However, not all of these patients experienced disease remission, suggesting that their tumors were hormone insensitive rather than that the dose of 4-OHA was suboptimal. In trials of patients who had not received prior tamoxifen treatment, 4-OHA (250 mg i.m. every 2 weeks) was found to induce complete or partial tumor regression in 33% of patients. The response of patients was not significantly different from that observed in patients treated with tamoxifen (30 mg o.d) of 37%. No significant difference between treatments was observed for disease stabilization, the duration of response or median survival. Several other steroidal aromatase inhibitors have been studied, such as 7α-substituted androstenedione derivatives. MDL 18962 [10-(2-propynyl)estr-4-ene-3,17-dione] and FCE 24304 (6-methylen-androsta-1,4-diene-3,17-dione) are currently in clinical trials. Non-steroidal inhibitors of cytochrome *P*-450 enzymes, such as imidazole and triazole derivatives have been developed which are highly selective for aromatase. Three triazoles which are very potent and selective inhibitors are vorazole (6-[(4-chlorophenyl)(1H-1,2,4-triazol-1-yl)-methyl]1-methyl-1H-benzotriazole R 76713, arimidex 2,2′[5-(1H-1,2,4-triazol-1-yl methyl)-1,3-phenylene]bis(2-methylpropiononitrile) (ZD1033) and letrozole 4-[1-(cyanophenyl)-1-(1,2,4-triazolyl)methyl]benzonitril (CGS 20267). These compounds reduce serum estradiol concentration to undetectable levels in breast cancer patients. These highly potent inhibitors provide the opportunity to determine whether a further degree of estrogen suppression will be important in producing greater clinical response. With the recent approval of 4-OHA in several countries and the introduction of the potent new compounds, aromatase inhibitors either alone or in combination with the antiestrogen are likely to improve the treatment of breast cancer.

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INTRODUCTION

The traditional methods for the treatment of breast cancer have been surgical ovariectomy and adrenalectomy to remove the major sources of estrogens and their precursors. These procedures are associated with some morbidities and mortalities and are not highly effective in all patients. More recently, many studies have indicated a correlation between the mitogenic and proliferative actions of estrogens and concentrations of estrogen and progesterone receptors in breast tumor tissue [1]. By utilizing such measurements to identify patients with estrogen-sensitive tumors, treatment methods which use pharmacological approaches to reduce the effects of estrogen are proving more effective. There are two classes of compounds that are currently being used. One class consists of

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antiestrogens which interact with estrogen receptors in the tumor cells to block the action of estrogens [2]. The other class comprises compounds that block estrogen synthesis via inhibition of the enzyme aromatase (estrogen synthetase). Although selective aromatase inhibitors were identified [3] and their antitumor effects first reported a number of years ago [4], they have only recently been evaluated in breast cancer patients [5, 6]. During this time, the antiestrogen tamoxifen has been found to extend the disease-free interval and significantly increase survival of postmenopausal breast cancer patients [7]. Tamoxifen is a weak agonist as well as an antagonist and in spite of the benefits of this antiestrogen, breast tumors may eventually become resistant to the drug and lead to disease recurrence in the patient. In contrast, inhibitors of aromatase do not have estrogenic activity and may thus be able to achieve total estrogen blockade. Furthermore, their different mode of action from antiestrogens may improve patients' response when used in combination with tamoxifen, as well as being effective in some tamoxifen-resistant patients with advanced disease [5, 6]. The use of tamoxifen as adjuvant therapy in patients at early stages of the disease and aromatase inhibitors for treatment of recurrences may be a more effective strategy which could provide additional advantages and a longer duration of response.

AROMATASE

Aromatase is a cytochrome *P*-450 hemoprotein with approx. 30% homology to other cytochrome *P*-450 enzymes [8]. The region of greatest homology among all steroidogenic *P*-450 enzymes is the heme binding region. However, because the low overall homology of aromatase to other members of the *P*-450 superfamily, the gene encoding $P\text{-}450_{arom}$ belongs to a separate gene family designated CYP19 [9]. The *P*-450 genes vary widely in overall size although they contain 8–10 exons of similar size. The 21-hydroxylase is approx. 3 kb while that of $P\text{-}450_{arom}$ is the largest with more than 70 kb. However, the region encoding the 55 kDa protein of 503 amino acids spans about 35 kb of DNA and contains 9 exons (II–X). Exon II contains the translation initiation site. The $P\text{-}450_{arom}$ gene has been mapped to chromosome 15 [10]. Aromatase transcription is highly regulated and its control is tissue specific with at least four major promoter sites which respond to gonadotropins, cyclic nucleotides, glucocorticoids, phorbol esters, growth factors and cytokines. Aromatase messenger RNAs have major differences in their untranslated regions, whereas the translated regions of the aromatase polypeptide are identical in all tissues [11].

Steroidogenic *P*-450 enzymes function as hydroxylases in the conversion of one steroid to another. The conversion of androgens to estrogens appears to involve three hydroxylation steps mediated by $P\text{-}450_{arom}$ [12]. The first hydroxylation occurs at the C-19 of the androgen substrate [13, 14]. The 19-hydroxylated intermediate then hydrogen bonds to an acidic side-chain residue Glu-302 [15, 16] within the enzyme's active site which is thought to be of critical importance in the process of aromatization [17]. Hydrogen bonding of the 3-ketone may also occur at a polar active site (His-128 residue) to anchor the intermediate. This assures stereospecific removal of the C-19 pro-R hydrogen by a heme iron-oxo species during the second hydroxylation step. The ferric peroxide breakdown which then usually occurs may be circumvented because of the high electrophilicity of the aldehyde. This would alter the normal hydroxylation cycle [18]. The mechanisms involved in the last step are less clear and have resulted in a number of hypotheses being proposed over the years. However, cumulative evidence [17, 19, 20] supports the idea that the unstable intermediate produced by the series of hydroxylations collapses yielding estrogens and formic acid. This last step is postulated to involve hydride shift, proton transfer or free-radical pathways [21] resulting in the removal of the angular methyl group at C-19, *cis* elimination of the 1β and 2β hydrogens [22], and aromatization of ring A of the androgens to form the estrogens.

AROMATASE INHIBITORS

Although hydroxylations characteristic of steroidogenic *P*-450 enzymatic reactions are involved in conversion of androgens to estrogens, Brodie and coworkers [3] proposed that because of some of the unique features in the aromatic ring formation discussed above, selective aromatase inhibition might be achieved with substrate analogs. In addition, aromatization of androgens is the last step in the biosynthetic sequence of steroid production. Thus, selective blockade of aromatization will not interfere with the production of other steroids, such as adrenal corticoids. For these reasons, aromatase is a particularly suitable enzyme target for selective inhibition.

A number of androstenedione derivatives including 4-hydroxyandrostenedione (4-OHA), were identified as potent and selective aromatase inhibitors [4, 23–25]. Further studies with 4-OHA and other androstenedione derivatives revealed that several of these inhibitors appear to be functioning as mechanism-based inhibitors and cause inactivation of the enzyme [26, 27]. While not intrinsically reactive, the compounds compete with the substrate and presumably are converted by the enzyme to a form which binds either very tightly or irreversibly at the active site [28]. Inhibitors of this type are therefore usually quite specific. Also, as the enzyme is inactivated, the continued presence of the drug to maintain inhibition is unnecessary, thus reducing the chances of toxic side-effects. However, the effectiveness of this type of

inhibition *in vivo* is dependent on the rate of new enzyme synthesis.

There are a number of other steroid analogs in addition to 4-OHA which have been reported to cause inactivation of aromatase and are effective in reducing plasma estrogen levels *in vivo*. Of several C-19 acetylenic analogs of androstenedione designed to be mechanism-based or enzyme-activated inhibitors [29], 10-(2-propynyl)estr-4-ene-3,17-dione (MDL 18962) is reported to be the most potent aromatase inhibitor of this series of analogs. The compound has significant and lasting biochemical and pharmacological activity *in vivo*. Thus, the ED_{50} of MDL 18962 for aromatase inhibition 6 h after a single oral dose was 3 mg/kg in athymic mice bearing human trophoblastic xenografts [30]. In non-human primates, peripheral aromatization was inhibited by this compound for up to 1 week after a single i.v. pulse [31]. Henderson *et al.* [32] have shown that 1-methylandrosta-1,4-diene-3,17-dione (SH 489) causes inactivation *in vitro* and its use in the treatment of benign prostatic hypertrophy (BPH) is now being explored [33]. Two other aromatase inhibitors which cause enzyme inactivation and have demonstrated biological activity are 6-methylenandrosta-1,4-diene-3,17-dione (FCE 24304) [34] and 4-aminoandrosta-1,4,6-triene-3,17-dione (FCE 24928) [35]. It was recently reported that 14-hydroxyandrostene-3,6,17-trione causes inactivation of the enzyme, and inhibits ovarian aromatase and estrogen production in the rat [36].

Brueggemeier *et al.* [37, 38] have studied a number of 7α-substituted androstenedione derivatives, several of which cause aromatase inactivation. The covalent nature of the binding of 7α-(4′-amino)phenylthio-1,4-androstadienedione (7α-APTADD) to aromatase was demonstrated by several methods. These included extensive dialysis of the radiolabeled inhibitor [^{125}I]-7α-IPTADD bound to purified cytochrome P-450_{arom}. The dialyzed solution was then subject to affinity column purification, electrophoresis/autoradiography, protein precipitation on glass fiber filters and HPLC. Separation of fragments from tryptic digestion of the [^{125}I]7α-IPTADD–aromatase complex indicated that the inhibitor was bound to the lipophilic fragment with a retention time of 42 min on HPLC [39].

Despite the potent activity of some of the early aromatase inhibitors, it was a number of years before it was possible for 4-OHA to reach clinical development. However, about the time of the initial studies on the antitumor activity of aromatase inhibitors, aminoglutethimide, a compound developed for the treatment of epilepsy and later found to inhibit adrenal steroidogenesis, was being used in postmenopausal breast cancer patients to produce "medical adrenalectomies". Subsequently, Santen and co-workers [40] observed that although plasma estrogen concentrations were reduced in these patients normal androgen levels were maintained. This suggested that aminoglutethimide has a more pronounced effect on aromatase than on other P-450 steroidogenic enzymes. Aminoglutethimide was then used to test the concept of aromatase inhibition for the treatment of breast cancer, although glucocorticoid replacement therapy was usually provided to the patient.

Although aminoglutethimide was found to be effective therapy for breast cancer, its lack of specificity and potency and its significant side-effects limited its usefulness. Attempts were therefore made to develop more potent and specific analogs [41, 42]. In addition, a number of other non-steroidal aromatase inhibitors such as imidazole derivatives are being investigated. These are related to antifungal agents such as ketoconazole, which act by inhibiting fungal cytochrome P-450 enzymes. The imidazole derivatives have been shown to be quite selective as well as potent aromatase inhibitors. Such compounds include fadrozole [4-(5,6,7,8-tetrahydro-imidazo-[1,5a]-pyridin-5-yl)-benzonitrile monohydrochloride (CGS 16949A) [43] and (3αR)-*trans*-1-[(3α-ethyl-9-(ethylthio)-2,3,3α,4,5,6-hexahydro-1H-phenalen-2-yl)-methyl]-1H-imidazole HCl) (ORG 33201) [44].

Besides the imidazoles, the structurally related N-substituted triazoles, which are also antifungal agents, have served as a basis for aromatase inhibitor design. Greater selectivity for P-450_{arom} appears to be achieved with these non-steroidal inhibitors by interaction as the sixth ligand with the heme iron atom and also interaction with amino acid residues located near to the heme site [45]. Studies on the interaction of R 76713 (vorazole)(6-[4-chlorophenyl)(1H-1,2,4-triazol-1-yl)methyl]1-methyl-1H-benzotriazole with aromatase determined that this triazole derivative coordinates with the heme iron and that its N-1-substituent occupies a lipophilic region of the apoprotein moiety of the P-450. This appears to be an area formed by a gap between helix-B′ and the loop K473–L77 involving residues I-126 and I-474. This pocket is not filled by the natural substrate and extends from C6–C7 of the steroid.

In addition to R 76713 [46] there are two other triazole derivatives now in clinical trials that are very potent and selective aromatase inhibitors. They are Arimidex 2,2′[5-(1*H*-1,2,4-triazol-1-yl methyl)-1,3-phenylene]bis(2-methylpropiononitrile) (ZD 1033) [47] and letrozole 4-[1-(cyanophenyl)-1-(1,2,4-triazol-yl)methyl]benzonitril (CGS 20267) [48]. Triazole antifungal agents administered systematically are more slowly metabolized than imidazoles. This may account for the finding that although CGS 20267 inhibits aromatase to the same extent as fadrozole hydrochloride (CGS 16949A) *in vitro*, it is 10 times more potent *in vivo* in the rat. In the human, CGS 20267 is about 100 times more potent than CGS 16949A in reducing serum estradiol levels, probably reflecting the significantly longer half-life of CGS 20267 than of fadrozole. Other *in vivo* studies with CGS 20267 indicate that it

Table 1. The effect of 4-OHA on postmenopausal breast cancer patients with advanced disease

Trial	Patients eval./total	Injected dose/time	CR	PR	NC	PD
Coombes *et al.*	72/96	250 mg/2 weeks	3	14	15	40
	24/29	500 mg/2 weeks	1	6	6	11
	40/61	500 mg/week	2	9	7	22
Höffken *et al.*[a]	86/91	500 mg/2 weeks (6w) 250 mg/2 weeks	2	19	26	39
Pickles *et al.*	18/22	250 mg/2 weeks	0	7	5	6
Total evaluated	240		3.3%	22.9%	24.6%	49.2%

[a]Patients received 500 mg/2 weeks 4-OHA im for 6 weeks, then 250 mg/2 weeks thereafter.
CR = complete response, PR = partial response, NC = no change, PD = progressive disease.

lacks significant toxicity and is without appreciable effect on the other steroidogenic enzymes, cholesterol side-chain cleavage, 11-hydroxylase, 21-hydroxylase or 18-hydroxylase [48, 49].

CLINICAL STUDIES WITH AROMATASE INHIBITORS

One of the first selective aromatase inhibitors identified in our early work was 4-OHA. This compound has now been studied in a number of trials in breast cancer patients during the last few years [5, 6]. The data from three recent trials in which 4-OHA (®Lentaron, formestane) (CGP 32349) was evaluated in 465 breast cancer patients are shown in Table 1 [50]. The patients received either 500 mg by intramuscular (i.m.) injection weekly or biweekly, or 250 mg biweekly. Injections of 4-OHA i.m. appeared to be well-tolerated and had notably less toxicity than AG. Side-effects occurred in 17% of patients and were mostly mild, only 3–5% of patients discontinued treatment. Local reactions, usually sterile abscesses, occurred in a small percentage of the patients (<10%) [51–53] and were a feature mainly of the higher injected dose (500 mg). The overall results of the studies indicate that 4-OHA is effective in postmenopausal breast cancer patients with advanced metastatic disease who have relapsed from previous hormonal therapy, usually tamoxifen. Response rates were not significantly different between the two doses or frequency of administration. Thus, 28% of patients experience complete or partial regression of their tumors, while the disease was stabilized in an additional 22% of patients. The disease progressed in the remaining women. When 250 mg/day 4-OHA was administered orally, the response rates of patients were similar to those of patients receiving biweekly injections [54].

Serum estradiol levels in all patients were suppressed by 4-OHA without apparent differences between regimens and were maintained for at least several months [55]. It therefore seems likely that the lack of response of some patients to treatment may be due to hormone insensitivity of the tumor rather than to suboptimal doses of 4-OHA. In those patients who had relapsed from tamoxifen but who responded to 4-OHA treatment, it appears that their tumors remained sensitive to estrogens. Some recent studies have suggested mechanisms by which tumors may become resistant to tamoxifen but retained hormone responsiveness. For example, Dowsett *et al.* [56] has recently found changes in the uptake of tamoxifen by tumors of some patients. In addition, human breast cancer cell lines treated with tamoxifen have been reported to have increased concentrations of estrogen receptor [57] or increased concentrations of progesterone receptor in subpopulations of cells which may thereby circumvent the effects of tamoxifen [58].

An international, multicenter, randomized trial carried out by the AH/BC 4 International Study Group has recently been completed in which 4-OHA was compared with tamoxifen in 409 previously untreated postmenopausal breast cancer patients [59]. Patients with measurable lesions according to modified UICC criteria were randomized into two groups which were well-matched for baseline characteristics and prognostic factors known to influence response. In both groups, approx. 40% of patients were known to have estrogen receptor positive tumors. The remaining patients were of unknown receptor status. The groups were also well-matched for previous surgery, adjuvant therapy, tumor burden and site of metastases. Tamoxifen was administered orally 30 mg o.d., whereas 250 mg 4-OHA was injected i.m. every 2 weeks. The treatments were equivalent in terms of response rates. Thus, in 348 evaluable patients complete or partial tumor regression occurred in 33% of patients treated with 4-OHA and in 37% of patients who received tamoxifen. A further 25–30% of patients achieved disease stabilization. There was no significant difference in the median duration of response between the two treatment groups, with a median duration of response for patients receiving ®Lentaron of 15 months. Furthermore, there was no significant difference in survival, with a median survival for patients receiving ®Lentaron of 33 months. Elderly patients (over 70 yr) with soft tissue metastases achieved higher response rates to both 4-OHA (42%) and tamoxifen (38%) than did younger patients with bony or visceral

involvement. The two treatments were well-tolerated and no evidence of increased toxicity was experienced by elderly patients. Pain or inflammation at the injection site which subsided within 48 h occurred in 7% of patients treated with 4-OHA. Nevertheless, this route of administration was considered advantageous by some patients, as it assured compliance and involved more frequent medical attention to their cancer, every 2 weeks.

Several other steroidal and non-steroidal aromatase inhibitors are now undergoing clinical evaluation. Phase 1 trials are in progress for steroidal inhibitors MDL 18,962, SH 489 and FCE 24304 as discussed above.

Fadrozole (CGS 16949A) is the first imidazole derivative to be studied in a large cohort of patients with advanced breast cancer [60]. In doses ranging between 2 and 4 mg/day, fadrozole suppressed plasma and urinary estrogen levels to the same extent as aminoglutethimide [61] but complete suppression was not achieved. However, patients experienced few side-effects with fadrozole, although adrenal cortisol and aldosterone secretion was blunted during ACTH stimulation tests [62, 63]. The triazole derivatives appear to be more specific. No effect on adrenal steroid synthesis was evident with ZD 1033 at doses of 5 and 10 mg [47]. In any case, serum estradiol concentrations were suppressed to the limit of assay detection with a dose of 1 mg per day. Vorazole (R 76713) and letrozole (CGS 20267) are also highly selective, potent inhibitors and are well-tolerated in patients. Of particular interest is suppression of estrogen levels in premenopausal women treated with R 76713. Plasma estradiol values fell from 389 pmol/l to a mean of 149 pmol/l over 4–24 h [64]. Recently, a Phase 1 study has been carried out with CGS 20267 in 8 postmenopausal breast cancer patients with metastatic disease [65]. Doses as low as 0.1 and 0.25 mg per day were administered orally to the patients for 12 weeks. Marked suppression of plasma estradiol, estrone and estrone sulfate levels occurred within 24 h of administration. Similar results had been observed in normal postmenopausal women [66]. During the following 12 weeks of treatment, estradiol concentrations in the patients were suppressed to undetectable levels despite the use of a highly sensitive assay [65]. No changes in the levels of cortisol or aldosterone occurred even after provocative stimulation with ACTH, nor in the urinary sodium and potassium values during the 12 weeks of treatment. Even in normal male subjects administered single doses of 0.02–30 mg CGS 20267, no changes in cortisol or aldosterone concentrations were found [67]. These results suggest that this compound is a very powerful and selective aromatase inhibitor *in vivo*.

FUTURE PERSPECTIVES

It is now apparent that the steroidal aromatase inhibitor 4-OHA has a place as second line treatment in breast cancer patients relapsing from tamoxifen. This compound is approved for use in the U.K. and more recently in a number of other countries as well. Several of the newer inhibitors entering clinical trials may also be effective in similar types of patients. In view of their different potencies and different mechanisms of interaction with the enzyme, it will be important to compare the effects of these aromatase inhibitors in patients of equivalent status. Also, since their main target is not within the cancer cell, resistance is unlikely to develop directly to aromatase inhibitors. Steroidal inhibitors may have additional biological activities mediated via steroid receptors (e.g. androgen receptors) which may elicit beneficial responses in patients, such as belief of bone pain. Studies in patients may reveal other activities of a compound which could influence its efficacy and tolerability. Several new non-steroidal compounds are highy potent and selective inhibitors. A further degree of estrogen suppression in women with breast cancer would be important in producing a clinical response. Whether complete suppression of estrogen production and action will result in significantly enhanced tumor regression remains to be determined. However, the potency of these newer aromatase inhibitors provides the opportunity to test this hypothesis in the near future. The variety of compounds inhibiting aromatase offers the possibility that a number of useful drugs will become available. Their actions alone or in combination with the antiestrogen are likely to improve the treatment of breast cancer patients and other diseases influenced by estrogens.

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