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AROMATASE AND ITS INHIBITORS—AN OVERVIEW

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Summary—Estrogen synthesis by aromatase occurs in a number of tissues throughout the body. Strategies which reduce production of estrogen offer useful means of treating hormone-dependent breast cancer. Initially, several steroidal compounds were determined to be selective inhibitors of aromatase. The most potent of these, 4-hydroxyandrostenedione (4-OHA) inhibits aromatase competitively but also causes inactivation of the enzyme. A number of other steroidal inhibitors appear to act by this mechanism also. In contrast, the newer imidazole compounds are reversible, competitive inhibitors. *In vivo* studies demonstrated that 4-OHA inhibited aromatase activity in ovarian and peripheral tissues and reduced plasma estrogen levels in rat and non-human primate species. In rats with mammary tumors, reduction in ovarian estrogen production was correlated with tumor regression. 4-OHA was also found to inhibit gonadotropin levels in animals in a dose-dependent manner. The mechanism of this effect appears to be associated with the weak androgenic activity of the compound. Together with aromatase inhibition, this action may contribute to reducing the growth stimulating effects of estrogen. A series of studies have now been completed in postmenopausal breast cancer patients treated with 4-OHA either 500 mg/2 weeks or weekly, or 250 mg/2 weeks. These doses did not affect gonadotropin levels. Plasma estrogen concentrations were significantly reduced. Complete or partial tumor regression occurred in 26% of the patients and the disease was stabilized in 25% of the patients. The results suggest that 4-OHA is of benefit to postmenopausal patients who have relapsed from prior hormonal therapies. Several of the steroidal inhibitors are now entering clinical trials as well as non-steroidal compounds which are more potent and selective than aminoglutethimide. Aromatase inhibitors should provide several useful additions to the treatment of breast cancer.

INTRODUCTION

We began to design compounds that would be selective inhibitors of estrogen biosynthesis in the early 1970s [1] proposing that effective inhibitors could be useful as tools for research and as treatment for a number of conditions in which estrogens are involved. Because of the important role of estrogen in breast cancer, we focused much of our efforts on this application [2, 3].

Studies on estrogen production indicate that synthesis occurs not only in the ovaries but in several tissues throughout the body. For example, aromatase in the brain of many species may have a role in controlling sex differentiation as well as in reproduction [4]. The testes of the horse have the highest production of estrogen of any tissue, although these appear mainly as estrogen 3-sulfates in testicular venous blood [5]. In postmenopausal women, extraovarian production is the most important source of estrogens [6]. Breast cancer is more prevalent in this age group than in younger women and about 75% of patients have hormone-responsive tumors, compared to about 60% of premenopausal patients [7]. In the former group, aromatase has been detected in the tumor [8–10] and could be an additional source of estrogens which can act locally to stimulate tumor growth. Our rationale for breast cancer treatment was therefore to inhibit estrogen production in all tissues.

At the time when we began this work, all antiestrogens which block the action of estradiol at the receptor site were known to be weak or partial agonists. Therefore, another important goal of our research on aromatase inhibitors was to identify compounds without estrogenic activity. Because of its partial agonist activity, tamoxifen may not be the optimal antiestrogen. Nevertheless, tamoxifen has now been shown to provide better response rates and less toxicity than cytotoxic agents in postmenopausal patients with estrogen receptor-positive breast cancer [11]. However, as is the case with most anticancer agents, patients eventually relapse from tamoxifen treatment. Thus, aromatase inhibitors could provide a second course of

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treatment for these women. Furthermore, aromatase inhibitors may be more effective than tamoxifen for the reasons stated above, or have a longer duration of action. An additional objective of our research was to identify agents with low toxicity that could be utilized over an extended period in a more advantageous strategy, i.e. adjuvant therapy. As tamoxifen is now being used more and more in this role, aromatase inhibitors may have a place as first line therapy in recurrent disease.

The aromatase complex consists of a cytochrome *P*-450 hemoprotein and a flavoprotein, NADPH-cytochrome *P*-450 reductase. The latter is common to most cell types, and functions to donate electrons to the cytochrome *P*-450. The *P*-450 aromatase (P-450_{arom}) binds the C-19 androgen substrates, androstenedione and testosterone and catalyzes their conversion to estrone and estradiol. This reaction appears to involve three hydroxylations [12], loss of the angular methyl group at C-19 and *cis* elimination of 1β and 2β hydrogens which results in the aromatization of ring A of the androgens to form the estrogens [13–15]. Aromatization is unique to estrogen synthesis and furthermore is the last step in the biosynthetic sequence of steroid production. We envisaged that these features may provide selectivity to inhibitors reacting with the enzyme.

INHIBITORS INACTIVATING AROMATASE

Following our initial report [1], we identified 4-hydroxyandrostenedione, as well as several other steroidal derivatives, as potent aromatase inhibitors [3, 16, 17]. Although these compounds exhibit properties typical of competitive inhibitors, some also caused inactivation of the enzyme [18]. These compounds are thought to be functioning as mechanism-based inhibitors. While not intrinsically reactive, they initially compete rapidly with the enzyme's natural substrate and subsequently interact with the active-site of the enzyme, binding to it either very tightly or irreversibly and causing its inactivation. Potentially, compounds of this type should have long lasting effects *in vivo* so that the continued presence of the drug is not required, thus reducing the chance of toxic side effects. In addition, they should be quite specific since they interact with the active-site of the enzyme. Inactivation of aromatase by 4-OHA was demonstrated by preincubating the compound for various lengths of time with microsomes of human placenta or rat ovaries in the presence of NADPH. After removal of the compound, a time-dependent loss of enzyme activity was observed which followed pseudo-first order kinetics [18]. In addition, the binding of [6,7^{3}H]4-OHA to placental microsomes appears to be specific and irreversible (unpublished data). A number of other steroidal aromatase inhibitors have also been reported to cause inactivation and appear to be acting by the same mechanism eg. 10(2-propynlestr-4-ene-3,17-dione [19] and analogs of 7α-(4′-amino)phenylthio-androstene-3,17-dione [20]. Brueggemeier *et al.* have shown recently that radiolabeled 7-substituted androstenediones caused inactivation of the purified human placental cytochrome P-450_{arom} [21]. Two new compounds 6-methylen-androsta-1,4-diene-3,17-dione (FCE 24304) and 4-aminoandrosta-1,4,6-triene-3,17-dione (FCE 24928) are also reported to inactivate aromatase. Following oral administration of FCE 24304 to rats, plasma estrogen levels remained depressed after 24 h [22]. The time-course of inhibition of ovarian aromatase activity was found to be similar to that of the reduction in plasma estradiol levels, suggesting that FCE 24304 inactivates ovarian aromatase *in vivo*. Dowsett *et al.* [23] have recently investigated the pharmacokinetics of oral administration of 250 mg 4-OHA in breast cancer patients. The half-life of 4-OHA was found to be about 3 h. However, the initial serum concentrations of 4-OHA were quite high (averaging about 50 ng/ml from 30–90 min) relative to the serum levels of androstenedione (0.5 ng/ml) which did not change from pretreatment values (0.5 ng/ml). Although high concentrations of the substrate can protect an enzyme from inactivation, the above results suggest that the serum androstenedione concentration may be insufficient to protect aromatase from inactivation by 4-OHA. By 24 h, the plasma concentrations of 4-OHA were almost undetectable (<0.8 ng/ml). Nevertheless, progressive suppression of plasma estradiol levels continued during the first 24 h of treatment despite the rapid clearance of 4-OHA from the blood. These results suggest that irreversible inhibition of aromatase by 4-OHA may be occurring *in vivo*.

We have carried out a number of studies in animal models which demonstrate that 4-OHA inhibits ovarian estrogen production and aromatase activity. These effects are correlated with marked regression of DMBA-induced,

hormone-dependent, mammary tumors in the rat [3, 24]. Because of the importance of peripheral aromatase in postmenopausal breast cancer patients, we also investigated whether 4-OHA inhibits peripheral aromatization. Using male rhesus monkeys, aromatization of [7-^{3}H]androstenedione to estrogens was undetectable in 3 of 4 animals and markedly suppressed in the fourth monkey [25]. Recently, inhibition of peripheral aromatization was confirmed by Reed *et al.* [26] who performed similar studies in breast cancer patients administered a dose of 4-OHA that produced significant tumor response. Aromatase activity in the breast tumor of these patients was also found to be inhibited in 3 out of 7 patients, was unchanged in 2 out of 7 patients while in 2 out of 7 patients aromatase appeared to be resistant to the effects of 4-OHA. A decrease in DNA polymerase-α was noted in most tumors in which aromatase activity was inhibited. However, the correlation between aromatase activity and this marker of proliferation was not statistically significant. Although there are a number of reports of aromatase activity in breast tumors [8–10], the contribution of tumor aromatase to growth stimulation still requires further clarification.

COMPETITIVE, REVERSIBLE INHIBITORS

In addition to the steroidal derivatives, aromatase may be inhibited by compounds such as aminoglutethimide (AG) and several imidazole antimycotic agents. These compounds have in common a heteroatom which binds to the heme iron of the cytochrome *P*-450, inhibiting its action. Other cytochrome *P*-450 enzymes may also be similarly inhibited, such as those involved in adrenal production of aldosterone (18-hydroxylase) and cortisol (11β-hydroxylase) as is the case with AG [27]. This compound was used for a number of years to inhibit steroid biosynthesis in breast cancer patients and produced favorable responses [28]. Subsequently, it was observed by Santen and colleagues [29, 30] that androstenedione levels were preserved while estrone levels were reduced in the patients treated with AG, possibly due to compensatory increases in ACTH. Also, conversion of 5-ene to 4-ene steroids is enhanced [31]. This finding indicates that the main mechanism of AG in lowering estrogen levels may be aromatase inhibition. An additional effect of AG in patients appears to be a reduction of plasma levels of estrone sulfate by increasing its metabolism [32]. This action may occur as a result of induction of hepatic mixed function oxidases by AG [33]. Estrone sulfate may be an important source of estrogen within breast tumors [34]. Estrone sulfate is thought to be derived from circulating estrone and estradiol [35]. Therefore, aromatase inhibitors would be expected to be effective in inhibiting the production of estrone sulfate also. However, it is unclear why plasma estrone and estradiol levels are reduced only 50% of pretreatment values by AG [30, 36], whereas there is almost complete inhibition of peripheral aromatization of androstenedione to estrone.

Recently, analogues of AG, such as 3-ethyl-3-(4-pyridyl)piperidine-2,6-dione have been developed. While this compound is not more potent than AG, it appears to be rather more specific and less toxic [37, 38]. Some new imidazoles, such as CGS 16949A [39, 40], CGS 20267 [41] and R76713 [42, 43] appear to be highly potent inhibitors of aromatase. These compounds are reversible competitive inhibitors of aromatase. Because of their increased potency for this enzyme, they are more specific than AG.

OTHER, DOSE-RELATED PROPERTIES OF AROMATASE INHIBITORS

A further distinction between the effects of the two classes of compounds are apparent because of differences in the physiological regulation of gonadal and peripheral aromatase. Although it is currently believed that a single gene encodes only one species of aromatase in the human [44], regulation of the enzyme appears to be tissue-specific. Ovarian aromatase is regulated by gonadotropins but these hormones do not affect extragonadal aromatase. Regulation of aromatase in peripheral tissues is less clear, except for adipose tissue where glucocorticoids and growth factors have been found to be involved [45]. In studies carried out in premenopausal breast cancer patients, it was noted that ovarian production of estradiol could not be consistently suppressed by AG [46]. Initially, estrogen levels were reduced but subsequently there were reflex increases in the gonadotropins LH and FSH. Since these hormones stimulate ovarian steroidogenesis, the effect of AG blockade was counteracted, resulting in plasma estrogen concentrations similar to the pretreatment levels. These findings were confirmed in the rat model [47]. However, by contrast to treatment with AG, estradiol and

gonadotropin levels were suppressed by treatment with 4-OHA. Independent of its action on aromatase, 4-OHA was found to inhibit directly gonadotropin secretion in a dose-dependent manner. Thus, low ovarian estradiol production can be maintained during long-term treatment. We have also demonstrated that 4-OHA suppresses plasma LH, estradiol and progesterone levels in a premenopausal, non-human primate model (the baboon) when administered over 2 or 3 months [48]. Whether this mechanism could be an advantage which would enable 4-OHA or other steroidal inhibitors to be effective in premenopausal patients remains to be determined. Inhibition of gonadotropins has not been observed with the doses used to treat postmenopausal breast cancer patients [23].

Recent data indicates that maximum suppression of estradiol levels in postmenopausal patients occurs at daily doses of 2–4 mg of the imidazole CGS 16949A. However, doses of 8–16 mg daily appears to inhibit C11β- and C21-hydroxylases with increases in ACTH at the high dose level suggesting an effect on cortisol biosynthesis [49]. Inhibition of C11β-hydroxylase was confirmed *in vitro* in isolated adrenal cells [50]. At 16 mg daily CGS 16949A also blocks the corticosterone methyl oxidase type II step increasing the ratio of plasma 18-hydroxycorticosterone to aldosterone as well as urinary tetrahydro compound A to tetrahydroaldosterone [51]. At lower doses basal cortisol and ACTH levels were unaffected. However, cortrosyn-stimulated aldosterone levels were significantly blunted [52]. Thus, for the treatment of postmenopausal patients these effects may not be a problem.

CLINICAL RESULTS IN BREAST CANCER PATIENTS

4-OHA has now been evaluated in three recently completed trials of 465 breast cancer patients [53]. The results are summarized in Table 1. Initially we prepared a supply of 4-OHA and began studies in breast cancer patients in collaboration with Dr Charles Coombes then at the Royal Marsden Hospital, London [54, 55]. Subsequently, studies were continued using material prepared by Ciba-Geigy (CGP 32,349). The overall results of the three 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. The patients received either 500 mg i.m. weekly or biweekly, or 250 mg biweekly. The response rates were not significantly different between the different doses and frequency of administration. Overall, 26% of patients experience complete or partial regression of their tumors, while in a further 24.6% of patients the disease was stabilized. The disease progressed in the remaining women [53]. These results were similar to earlier reports by these groups when smaller numbers of patients had been studied [56–58]. The response rates among patients receiving 250 mg daily orally were also similar [59]. 4-OHA appeared to be well tolerated and had notably less toxicity than AG. Side effects occurred in 17% of patients and were mostly mild. Local reactions, including sterile abscesses were a feature mainly of the higher injected dose (500 mg) in a small percentage of the patients ($<10\%$). A small number of patients, approximately 3–5% discontinued treatment [56, 57].

Although in all of the studies a significant proportion of the patients were of unknown receptor status rather than estrogen-positive, the response rate to 4-OHA was similar to that with AG in previously treated patients [60, 61]. All regimens were effective in reducing serum estradiol levels to a similar extent. No escape of estradiol suppression from the treatment was observed in a group receiving 500 mg i.m. once a month, which was studied over a 4-month period [62]. Therefore, it appears that treatment failure in some patients is more likely to be due to hormone-insensitivity than to suboptimal

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

Trial	Patients eval./total[a]	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.*[b]	86/91	500 mg/2 weeks (6 weeks) 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%

CR Complete response; PR partial response; NC no change; PD progressive disease.
[a]Evaluated patients/total patients. [b]Patients received 500 mg/2 weeks 4-OHA i.m. for 6 weeks, then 250 mg/2 weeks i.m. thereafter.

doses of 4-OHA [57]. It is interesting that patients who have relapsed from tamoxifen respond to aromatase inhibitor treatment. The possibility that their tumors have become *super*sensitive to estrogens is suggested by the work of Jordan and colleagues who found that MCF-7 cells which had become resistant to tamoxifen contained increased concentrations of ER [63].

In addition to 4-OHA and CGS 16949A, other aromatase inhibitors are now entering clinical trials. It is apparent that 4-OHA and possibly the newer compounds will have a place as second line treatment in patients relapsing from tamoxifen. 4-OHA could also be useful as first line treatment in patients who progress to advanced disease following adjuvant therapy with tamoxifen. It remains to be determined how these compounds will compare with one another in view of their different mechanisms; competitive, reversible inhibition versus enzyme inactivation as well as steroidal structures versus non-steroidal compounds. Other mechanisms of action which could affect the efficacy and tolerability of a compound may come to light during clinical evaluation. A variety of useful compounds should advance the prospects of improving treatment for breast cancer patients.

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