J. steroid Biochem. Vol. 27, No. 4–6, pp. 899–903, 1987
Printed in Great Britain.

0022-4731/87 $3.00 + 0.00

Proceedings of the VII International Congress on Hormonal Steroids (Madrid, Spain, 1986)

AROMATASE INHIBITORS: BASIC AND CLINICAL STUDIES

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Summary—Application of aromatase inhibitors to the treatment of conditions in which estrogen plays, a role is discussed. Studies *in vitro* demonstrate that 4-hydroxyandrostenedione (4-OHA) is a potent inhibitor of aromatase. The compound reduces ovariant estrogen production and causes regression of carcinogen (DMBA)-induced mammary tumors in the rat. In the rhesus monkey, 4-OHA was also shown to inhibit peripheral aromatization. To date 58 postmenopausal breast cancer patients with advanced metastatic disease have received 500 mg im weekly while 31 patients received 250 mg 4-OHA orally per day. Estradiol levels were significantly reduced in all patients from a mean of 7.2 + 0.8 pg/ml to 2.8 + 0.3 pg/ml. Of patients receiving 4-OHA im 27% had partial or complete responses and in 10% of patients the disease was stabilized. Similar responses occurred in the patients receiving 4-OHA orally. These results suggest that 4-OHA is effective and that this compound and other aromatase inhibitors could be valuable new additions to the treatment of breast cancer.

INTRODUCTION

The presence of aromatase in a variety of extragonadal tissue, in addition to the reproductive tissues of both males and females, provides sources of estrogen which may have important local effects and which are under different regulatory control mechanisms. There are a number of clinical situations in which estrogens either in normal amounts or when overproduced, have a role in the pathogenesis of the disease. Thus, inhibition of estrogen biosynthesis could be a valuable means of treating these conditions. For example, aromatase (estrogen synthetase) inhibitors could be useful for gynecomastia, endometriosis, precocious puberty, endometrial and breast cancer and possibly for contraceptive purposes.

A major emphasis in our development of aromatase inhibitors has been their potential for treatment of hormone-dependent breast cancer. Estrogen deprivation results in objectively quantifiable breast tumor regression which can often be long-lasting [1]. Use of cytotoxic therapy in metastatic breast cancer is not highly effective and the drugs have considerable toxicity. In the recent NIH consensus workshop, adjuvant treatment of postmenopausal breast cancer patients with the antiestrogen, tamoxifen, was concluded to be significantly superior to chemotherapy in extending disease-free interval for patients with steroid receptor positive tumors [2]. The response of premenopausal patients to tamoxifen was less clear. To date all antiestrogens are weak or partial estrogen agonists and thus may be insufficiently effective in blocking the action of the mid-cycle estrogen surge. Estrogen-controlling treatments are usually much less toxic than chemotherapy and can be used for long periods, in adjuvant therapy and in combination therapy regimens. For the above reasons, development of new strategies is warranted as alternatives or additions to current treatment.

Aromatase is an enzyme complex involving a NADPH-cytochrome c-reductase and a cytochrome P_{450} component which mediates the conversion of androgens to estrogens [3]. The reaction appears to involve three hydroxylation steps, two at the C-19 [4, 5] and one probably occurring at C-2 [6, 7] which result in the conversion of the A ring of the androgen molecule to an aromatic ring. Loss of the angular methyl group at C-19 and *cis* elimination of the 1β- and 2β-hydrogens yields estrogen and formic acid [8–10]. As aromatization is a unique reaction in the biosynthesis of steroids, it could provide some selectivity in the types of compounds which interact with the enzyme. In addition, since estrogens are the final products of steroid biosynthesis, aromatase inhibition *in vivo* should not cause deprivation of other essential steroids.

In 1973, our group reported the first of a number of steroid compounds which are selective inhibitors of aromatase [11]. Since that time, several highly active inhibitors have been identified [12, 13]. 4-Hydroxyandrostene-dione (4-OHA) is the most potent compound we have found and its *in vitro* and *in vivo* activity will be discussed in this chapter. This compound has low toxicity and is easily and cheaply synthesized. Clinical studies of 4-OHA in breast cancer patients have recently begun and the encouraging results to date indicate that the compound has significant antitumor activity [14].

As aromatization involves cytochrome P_{450} mediated hydroxylations, aromatase is also inhibited by compounds, such as aminoglutethimide (AG) [15, 16], which interfere with this component of the enzyme. Since AG had been used in the clinic as an anticonvulsant, it became the first compound to be evaluated in patients as an aromatase inhibitor. AG has been shown to inhibit extragonadal aromatization and to produce objective disease regression in breast cancer patients [17, 18]. However, it is less

potent and less specific than several steroidal compounds. It also exhibits significant side-effects and concomitant glucocorticoid replacement is required.

EVALUATION OF COMPOUNDS AS AROMATASE INHIBITORS *IN VITRO*

Over the past several years, we have evaluated a large number of compounds as inhibitors of aromatase [11, 19]. Candidate inhibitors were tested *in vitro* by comparing the extent of aromatization in incubations of microsomes prepared from aromatase-containing tissues. The conversion of androstenedione to estrogen by the microsomal preparation can easily be estimated by measuring the loss of tritium from the C-1β and C-2β positions [8] during aromatization of [1,2^{3}H(70%β]androstenedione. The tritium released as ^3H_2O is measured in the incubation medium after extraction of steroids by organic solvent [16]. First, human placental microsomes were used as the source of aromatase [11] and later a highly active microsomal preparation was developed from ovaries of rats stimulated with pregnant mares' serum gonadotropin (PMSG) [12]. Our studies of the two microsomal preparations suggest that subtle differences exist between enzymes from the two sources. The ovarian microsomal preparation was considered more appropriate for predicting *in vivo* activity in the rat.

The inhibitors with greatest activity in both systems are 4-hydroxyandrostene-3,17-dione (4-OHA) [20], 4-acetoxyandrostene-3,17-dione (4-acetoxyA) [21] and 1,4,6-androstatriene-3,17-dione (ATD) [22]. 4-OHA has been shown to have activity against human ovarian aromatase in granulosa cell cultures [23]. All show Lineweaver–Burk plots typical of competitive inhibition [11, 20] which occurs rapidly in the presence of both substrate (androstenedione) and inhibitor. 4-OHA, 4-acetoxy-A [24, 25], ATD and A-trione [22, 26] also cause slower time-dependent loss of enzyme activity which follows pseudo first-order kinetics in microsomes preincubated in the absence of substrate, but in the presence of NADPH. There was no loss of activity without added cofactors. Although 4-OHA caused the most rapid inactivation, the loss of activity with all three compounds was 10-fold slower in rat ovarian microsomes compared with the placental system. Aromatase activity was not regained after washed microsomes preincubated with 4-OHA had been allowed to stand for 18 h at 0°C followed by charcoal treatment and exhaustive washing to remove any residual inhibitor. These findings suggest that 4-OHA causes long-term inactivation and perhaps irreversible inhibition of aromatase.

Aromatase inactivation has also been reported to occur with the 10-propargyl analog of androstenedione [10-(2-propynl)estr-4-ene-3,17-dione] in the placental system [13, 26, 27] and was confirmed by us in both the placental and rat ovarian systems (unpublished observations). This compound was designed to inactivate aromatase by binding covalently to the enzyme. Other inhibitors synthesized by these investigators are allenes which probably lead to allene oxide intermediates via oxygen insertion by aromatase. The intermediate would alkylate either the prosthetic heme or surrounding enzymic protein causing inactivation of the enzyme [28, 29]. Although the precise mechanisms by which 4-OHA, 4-acetoxyA and ATD inactivate aromatase are unknown at present, their kinetics are similar to those of the 10-propargyl analog and suggest that they are k_{cat} or suicide inhibitors. Inhibitors of this type have been successfully developed as drugs for other enzymes. Since they bind to the active site of the enzyme, they are quite specific and have long-lasting effects *in vivo* due to inactivation of the enzyme [30].

Aromatase is inhibited approximately 4 times faster by 4-OHA than the 10-propargyl in *in vitro* preparations. On the other hand, the latter has a K_i of 4.5 nM with placental microsomes and has 2–3 times greater affinity for the active site of the enzyme than 4-OHA (K_i 10.2 nM) [28]. 16α-Bromoandrogens reported by Bellino *et al.*[31] and 7p-aminothiophenylandrostenedione synthesized by Counsell *et al.*[32] are also potent aromatase inhibitors but have not been studied in detail *in vivo*. Testololactone, a compound used for breast cancer treatment for over 20 yr [33], and subsequently reported to be an aromatase inhibitor [34] was found recently to cause aromatase inactivation, probably by virtue of the C-1 double bond [28]. However, similar to AG (K_i 770 μM), it has weak activity (K_i 750 μM, $t_{1/2}$ 21 min) in placental microsomes, compared to the above inhibitors [28].

IN VIVO EFFECTS OF AROMATASE INHIBITORS

Other pharmacological actions of compounds which are potent aromatase inhibitors may be important determinents of their effects *in vivo*. We have therefore done a variety of studies in animals to investigate whether significant reductions in ovarian and peripheral estrogen production can be achieved with 4-OHA and other inhibitors and the effect of active compounds on other parameters such as tumor growth.

1. *Inhibition of ovarian estrogen secretion*

Virgin female rats showing at least 2 consecutive regular 4-day cycles, were injected subcutaneously with inhibitor (50 mg/kg) at 1000 h on the day of proestrus. Blood (1 ml) was collected between 1400 and 1600 h by ovarian vein cannulation. The resulting plasma was then analyzed by radioimmunoassay to determine the concentration of estrogen. After collecting the ovarian vein blood, the ovaries were removed, homogenized and

aromatase activity measured from the tritium released during incubation of [1^{3}H]androstenedione. The most potent compounds in reducing ovarian estrogen secretion in the rat were found to be 4-OHA, 4-acetoxyA, ATD and AG. The reduction in ovarian aromatase activity was consistent with the reduction in estrogen secretion in animals treated with 4-OHA, AG or teslac [35].

Studies were also carried out in rats in which ovarian aromatase activity was stimulated by first priming the animals with PMSG over 11 days in order to overcome the normal cyclicity of the rat and maintain a constant estrogen secretion. On day 12, the animals were injected with 4-OHA or ATD. At various times after injection, blood was first collected from the ovarian vein, and then microsomes were prepared from the ovaries. Aromatase activity in the ovarian microsomes was reduced to 20% of the initial value within 8 h of injection and remained low for 24 h following injection of ATD and for 48 h following injection of 4-OHA. Estrogen concentrations in the ovarian vein samples were also reduced by inhibitor treatment and remained low for about the same length of time as aromatase activity. These findings suggest that enzyme inactivation is occurring in the ovary *in vivo* with both ATD [22] and 4-OHA [24] since activity could not be increased by procedures designed to remove unbound 4-OHA.

Similar results have been obtained using PMSG primed rats with the 10-propargyl compound [36].

2. *Antitumor activity of aromatase inhibitors in the 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary carcinoma model*

The DMBA-induced carcinoma model has been used extensively to study hormone-dependent mammary tumors. Although other hormones may be involved, the tumors are dependent on ovarian production of estrogen. In this regard, the model is comparable to the premenopausal breast cancer patient. Daily injections of 4-OHA (50 mg/kg per day) caused marked tumor regression [20]. Similar results were obtained with 4-acetoxyA, ATD [21, 22] and the 10-propargyl compound [36]. After 4 weeks of 4-OHA treatment, the total tumor volume in the experimental group was reduced by about 80% of the initial volume. At the end of 4 weeks of treatment, ovarian aromatase activity and estrogen secretion were both markedly inhibited compared with controls.

3. *Inhibition of peripheral aromatization*

Since extra-ovarian aromatization is an important source of estrogens in postmenopausal breast cancer patients, we also studied the effects of 4-OHA and 4-acetoxyA on peripheral aromatase by measuring the conversion of androstenedione to estrone during constant infusion of [7-^{3}H]androstenedione and [4-^{14}C]estrone. Male rhesus monkeys were used since most of the circulating estrogen in the male is of extragonadal origin. Each animal was infused under control conditions and during inhibitor treatment. 4-OHA was injected 18 h and then 3 h before the infusion was started. Peripheral aromatization was found to be undetectable in three of the four monkeys treated with 4-OHA and markedly reduced in the fourth animal. Treatment of two monkeys with 4-acetoxyA implants was also effective in reducing peripheral aromatization [37].

CLINICAL STUDIES WITH AROMATASE INHIBITORS

As already discussed, aminoglutethimide has now been used in a number of clinical trials and investigations. These have been reviewed by Santen *et al.* [17, 18]. AG is effective in postmenopausal breast cancer patients, and remission occurs in 40% of unselected patients. The compound is active in some patients who have relapsed from tamoxifen, indicating it can be used in addition to tamoxifen as well as an alternative treatment. Although somnolence is a side-effect of AG, recent studies with low-dose AG (500 mg/day) indicate it to be as effective as the higher dose (1 g/day) and is better tolerated [38].

Because of its greater potency *in vitro*, high efficacy in animal models, and ease of synthesis, 4-OHA was chosen for the first clinical evaluation with a selective aromatase inhibitor [14]. All patients were postmenopausal women with advanced metastatic disease. To date, 58 patients have received 500 mg 4-OHA intramuscularly weekly and 31 patients have received 250 mg 4-OHA orally each day for the first month, then 500 mg daily thereafter [39]. Plasma estradiol levels were suppressed from a mean of 7.2 ± 0.8 (SE) pg/ml before treatment to 2.6 ± 0.2, 2.7 ± 0.2 and 2.8 ± 0.3 mg/ml after 1,2 and >4 months, respectively, of treatment and remained suppressed in patients whose disease relapsed. There was slight but no significant decrease in estrone values. The treatment had no effect on gonadotropin levels, dehydroepiandrosterone sulfate and sex hormone binding globulin.

Overall evaluation of 52 assessable patients of the 58 (Table 1) revealed that 14 (27%) had objective, complete (2) or partial (10) responses to treatment. In 10 (19%) patients the disease stabilized for at least 8 weeks on therapy whereas the disease progressed in 28 (54%) patients. Six patients were not assessable as they had received treatment for less than 3 weeks. Twenty-two patients were estrogen receptor (ER) positive, 6 of these responded to 4-OHA, 3 had static disease and 13 experienced progression of the disease. Twenty-four patients had previously responded to endocrine therapy, and 7 of these responded to 4-OHA, while in 3 the disease stabilized. Responses seemed to occur most often in soft tissue and lymph nodes affected by breast cancer, with only one response in a visceral site. There were no responses in liver metastases of 11 patients affected. Four of 35

Table 1. Response to 4-hydroxyandrostenedione according to estrogen receptor status and previous response to endocrine therapy

	Response to 4-OHA				
	CR	PR	NC	PD	NA
Overall response	4	10	10	28	6
	14				
ER status					
positive	1	5	3	13	2
negative	0	1	0	2	0
unknown	3	4	7	13	4
Previous response to endocrine therapy					
Responders	2	5	3	14	3
Non-responders	2	2	3	9	0
No previous therapy or response not assessable	0	3	4	5	3

CR—complete response; PR—partial response; NC—no change; PD—progressive disease; NA—not assessable.

Fourteen patients responded to 4-OHA. Only one responder was known to have an ER-negative tumor. Four patients who had failed to respond to other therapies (tamoxifen in all cases) responded to 4-OHA.

(11%) patients with skeletal metastases responded, although bone pain was alleviated in 5 out of 8 patients with this symptom. Of the 14 patients who responded to 4-OHA, 4 have since relapsed at 3, 4, 4 and 13 months. Ten patients remain in remission for periods between 2 and 18 months. The mean duration of response and response to subsequent therapy cannot yet be adequately evaluated.

Eight (26%) of 31 patients receiving oral 4-OHA showed evidence of partial response and 4 (13%) showed stabilization of disease [40, 41].

There were few side-effects of treatment, the most significant being sterile abscesses and pain at the site of injection in about 10% of patients receiving 500 mg 4-OHA intramuscularly. Thus, this aromatase inhibitor reduces plasma estradiol levels and appears to be effective in patients who had previously relapsed from other endocrine therapies. The compound is well tolerated and has low toxicity. Further studies are required to determine the most effective dose and to compare efficacy with current standard treatments.

CONCLUSIONS

The foregoing studies indicate that aromatase inhibitors effectively reduce ovarian and peripheral estrogen production in animal models. In the rat, inhibition of ovarian estrogen secretion results in mammary tumor regression. In patients, AG and 4-OHA reduce peripheral estrogen levels in postmenopausal patients and cause significant tumor responses. However, 4-OHA is the first agent which selectively inhibits aromatase. This compound also has fewer side-effects than AG. The results emphasize the importance of extraovarian estrogen in postmenopausal breast cancer. From evidence reported at a recent NIH Consensus Conference, tamoxifen used in adjuvant therapy significantly extends the disease-free interval and overall survival of postmenopausal breast cancer patients. Aromatase inhibitors could be valuable new additions to breast cancer therapy.

Acknowledgements—This work was supported by NIH grants CA-27440 and HD-13909.

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