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AROMATASE AND OTHER INHIBITORS IN BREAST AND PROSTATIC CANCER

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Summary—Estrogens have an important role in the growth of breast and other hormone-sensitive cancers. We have shown that 4-hydroxyandrostenedione (4-OHA) selectively blocks estrogen synthesis by inhibiting aromatase activity in ovarian and peripheral tissues and reduces plasma estrogen levels in rat and non-human primate species. In postmenopausal men and women, estrogens are mainly of peripheral origin. When postmenopausal breast cancer patients were administered either by daily oral or parenteral weekly treatment with 4-OHA, plasma estrogen concentrations were significantly reduced. Complete or partial response to treatment occurred in 34% of 100 patients with advanced breast cancer, while the disease was stabilized in 12%. We recently studied the effects of 4-OHA and other aromatase inhibitors, 10-propargylestr-4-ene-3,17-dione (PED) and imidazo[1,5-α]3,4,5,6-tetrahydropyrin-6-yl-(4-benzonitrile) (CGS 16949A) as well as 5α-reductase inhibitors, N,N-diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxyamide (4-MA) and 17β-hydroxy-4-aza-4-methyl-19norandrost-5-en-3-one (L651190) in prostatic tissue from 11 patients with prostatic cancer and six patients with benign prostatic hypertrophy (BPH), and from normal men at autopsy. We attempted to measure aromatase activity in tissue incubation by quantitating ^3H_2O released during aromatization of androstenedione or testosterone labeled at the C-1 position. The amount of ^3H_2O released from all samples was at least twice that of the heat inactivated tissue samples. The ^3H_2O release was significantly inhibited by 4-OHA and 4-MA, but not by the other aromatase inhibitors. However, when HPLC and TLC were used to isolate steroid products, no estrone or estradiol was detected in the incubates. Furthermore, no aromatase mRNA was detected following amplification by PCR. The 4-OHA was found to inhibit 5α-reductase in both BPH and cancer tissue, although to a lesser extent than 4-MA. The other aromatase inhibitors were without effect. Although a mechanism involving intraprostatic aromatase is not likely, inhibitors may act to reduce peripherally-formed estrogens. In postmenopausal breast cancer, the results indicate that 4-OHA is of significant benefit.

INTRODUCTION

Estrogens have an important role in the growth of breast and other hormone-sensitive cancers. Synthesis of estrogens by the aromatase enzyme occurs in a wide variety of tissues in both males and females of many species and studies of the gene sequence of cytochrome $P450_{AROM}$ indicates that it is highly conserved [1]. We have recently investigated the location of the enzyme in sections of several human tissues by immunocytochemistry using a monoclonal antibody to purified $P450_{AROM}$ [2] using the peroxidase–antiperoxidase (PAP) staining or the biotin–avidin technique [3]. Our studies reveal that in the human premenopausal ovary, aromatase is located in the thecal compartment of the developing follicle, but as the follicle progresses towards ovulation, there is increased expression of the enzyme in granulosa cells [4]. In the testis, aromatase is known to perform a regulatory role in androgen biosynthesis. Immunocytochemical studies indicate that the enzyme is localized in the Leydig cells of the testis [5]. Aromatase has also been identified by conventional biochemical techniques in several locations in the brain [6], in adipose tissue of both men and women [7, 8] and in some breast tumors [9–12]. Thus, estrogens from many sources, both gonadal and extragonadal, can contribute to promoting the growth of estrogen sensitive cancers.

We have focused our research over the past several years on development of compounds which would reduce production of estrogens by inhibiting aromatase. We reasoned that

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inhibitors of aromatase by interacting with the enzyme in all tissues, could provide both selective and effective reduction of estrogen production and thereby be useful in the treatment of breast cancer. Recently, estrogen receptors have been identified in prostatic tissue [13, 14]. In men, the main source of circulating estrogens is from peripheral aromatization. However, greater than normal levels of estradiol have been detected in stroma of patients with benign prostatic hypertrophy (BPH) [15] and there are also reports suggesting that aromatase is present in the prostate [16]. These findings suggests that estrogens may have a role in this disease. If estrogens mediate the growth of prostatic tissue, aromatase inhibitors may also be useful in the treatment of diseases of the prostate.

AROMATASE INHIBITORS AND BREAST CANCER

Breast cancer is most prevalent among postmenopausal women; about 75% of patients have hormone-responsive tumors, whereas about 60% of premenopausal patients have hormone-responsive tumors [17]. Methods of controlling estrogen production or action result in objectively quantifiable breast tumor regression which can often be long-lasting [17]. Until recently, all inhibitors of estrogen action were also weak or partial agonists. Thus, tamoxifen may not be the optimal antiestrogen. It appears to be insufficiently effective in consistently blocking the action of the high levels of estrogen which occur in premenopausal women [18]. Aromatase inhibitors do not have agonistic activity and have the potential for being more effective than tamoxifen. Since we began our studies on aromatase inhibitors, it has become apparent that tamoxifen provides superior response rates and less toxicity than cytotoxic agents in postmenopausal patients with estrogen receptor-positive tumors [19]. Nevertheless, as with other anticancer agents, patients ultimately relapse from tamoxifen treatment. Aromatase inhibitors, therefore, offer an alternative approach to patients who have failed to respond to, or who have relapsed from tamoxifen treatment. In addition, a goal of our work was to find agents that have low toxicity and could therefore be used for long periods and in more advantageous strategies, such as an adjuvant therapy.

4-Hydroxyandrostenedione

In the early seventies, we began a program to synthesize and identify compounds which are potent aromatase inhibitors [20]. We envisaged that selective inhibition of aromatase was a feasible goal because aromatization of the androgen molecule is a unique feature of estrogen biosynthesis. In addition, since estrogens are the last steroids to be synthesized in the biosynthetic sequence, their inhibition would not interfere with the production of other essential hormones, such as cortisol.

The most potent inhibitor *in vitro* and *in vivo* that we have described is 4-hydroxyandrostene-3,17-dione (4-OHA) [21, 22]. Although this compound exhibited properties typical of competitive inhibitors, we also observed that it caused inactivation of aromatase. This phenomenon was demonstrated with 4-OHA by preincubating the compound for various lengths of time with microsomes from human placental tissue or rat ovaries in the presence of NADPH [23]. A time-dependent loss of enzyme activity was observed which followed pseudo-first-order kinetics and appears to be irreversible. Thus, 4-OHA is thought to be acting as a mechanism-based inhibitor. The compound is highly effective in reducing ovarian estradiol concentrations *in vivo* and causing regression of carcinogen (DMBA)-induced hormone-dependent mammary tumors in the rat. We have also demonstrated that the compound inhibits peripheral aromatization in non-human primates [24]. This finding has recently been confirmed in postmenopausal patients [25].

4-OHA is the first compound designed as a selective aromatase inhibitor to be evaluated for clinical use in breast cancer patients. One-hundred and twenty-eight postmenopausal patients with advanced, metastatic breast cancer have been treated with 4-OHA [26]. The first few patients received material prepared in our laboratories [27]. All subsequent patients received material supplied by Ciba–Geigy Pharmaceuticals, Basel, Switzerland (CGP 32349). Of the 128 patients treated with 4-OHA, 100 were assessable. Groups of patients received 500 mg i.m. once a week, 250 mg i.m. every 2 weeks or 500 mg orally every day. Patients were either postmenopausal or had been overectomized and had received 1–4 previous treatments. All had assessable locally advanced or metastatic disease. Assessment was made at 3, 6 and 9 months and at the end of treatment using

the criteria of the International Union Against Cancer. Of patients responding to treatment, 91% had estrogen receptor (ER)-positive tumors and of patients with disease stabilization 86% had ER-positive tumors. However, 53% of patients whose disease progressed had ER-positive tumors. Eighty of the 100 patients had received prior endocrine treatment (ovariectomy, tamoxifen, aminoglutethimide or megace). Patients who had previously responded to ovariectomy had an excellent (88%) chance of responding to 4-OHA. Some patients (24%) who failed to respond to previous treatments responded to 4-OHA. The compound was well-tolerated by the patients. The only major side-effect was local reaction at the site of injection in 13% of patients who received 500 mg i.m. Overall, 34% of patients experienced complete or partial response to treatment, the disease was stabilized in 12% but progressed in 54% of patients. The compound appears to be equally effective orally or by the parenteral route [26]. The efficacy of 4-OHA compared to tamoxifen in previously untreated breast cancer patients still remains to be determined.

Other steroidal and non-steroidal aromatase inhibitors

A number of other steroidal aromatase inhibitors have now been described. Some of these have also been reported to cause inactivation of aromatase and appear to be acting as mechanism based inhibitors e.g. 10(2-propynlestr-4-ene-3,17-dione (PED) [28] and analogs of 7α-(4′-amino)phenylthio-androstene-3,17-dione [29].

There are also several potent non-steroidal aromatase inhibitors, for example 4-(5,6,7,8-tetrahydrimidazo[1,5-α]pyridin-5-yl)benzonitrile monohydrochloride (CGS 16949A) [30] is an imidazole related to aminoglutethimide and 6[(4-chlorophenyl)(1*H*-1,2,4-triazol-1-yl)methyl]-1-methyl-1-*H*-benzotriazol (R76713) [31]. These compounds are very potent inhibitors *in vitro*. Thus, their actions on other enzymes, such as adrenal corticosteroids, are likely to be minor. These compounds appear to be competitive inhibitors of aromatase and do not cause its inactivation. Several of these promising inhibitors are now entering clinical trials.

STUDIES WITH PROSTATIC TISSUE *IN VITRO*

Search for aromatase

As there are reports of aromatase activity in tissue from patients with BPH [16], it was of interest to us to confirm this and to determine whether cancer tissue also contained the enzyme.

Tissues from 11 different patients with prostatic cancer and seven patients with BPH were investigated for the presence of aromatase. In this study, we attempted to use the ^3H_2O assay as an indirect assessment of estrogen production. This method depends upon the loss of hydrogen atoms at C-1β and C-2β positions of the androgen substrate during aromatization of ring A [32]. Thus, when substrates are labelled with tritium at these positions, ^3H_2O is formed equivalent to each mole of estrogen produced. The method has been widely used by ourselves and others to measure aromatization in the human placenta [22]. We had previously confirmed that the assay provides results equivalent to those of the product isolation method for measuring aromatization of androgens to estrogens in term placental microsomes [3]. However, we found that these two methods of measuring estradiol production did not provide equivalent results in the prostate. When prostatic tissue was incubated with [1β-^{3}H]androstenedione, significant release of tritium was observed compared to tissue which had been heat inactivated from the same patient. All values were at least twice the value of their corresponding tissue blank. In the first set of patients studied, the amount of tritium released was similar for both cancer tissue [mean $0.238 \pm 0.073\%$ (SE), $n = 6$] and for tissue from BPH patients [mean $0.281 \pm 0.047\%$ (SE), $n = 7$] [33]. Furthermore, the amount of tritium released to form ^3H_2O could be inhibited by aromatase inhibitor 4-OHA, suggesting that androstenedione was converted to estrogens. Values were reduced in all cancer tissue samples to $0.074 \pm 0.023\%$ and in all but one sample from BPH patients, the mean value was $0.100 \pm 0.067\%$. However, there was no significant inhibition by two other aromatase inhibitors, the steroidal compound PED, nor the non-steroidal aromatase inhibitor CGS 16949A. On the other hand, tritium release was inhibited by the 5α-reductase inhibitor, N,N-diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxyamide (4-MA). Several samples of prostatic tissue were also incubated with androgen substrates labeled at additional positions not involved in the aromatization reaction. In contrast to the implications of the above results, when either 13.5 μCi [1,2,6,7,^{3}H]androstenedione or 12.1 μCi [7^{3}H]testosterone was incubated and the products separated by HPLC, there was no evidence of conversion to tritiated

Table 1. Production of ^3H_2O from [1β-^{3}H]Androstenedione or estrogens from [7-^{3}H]testosterone by human prostatic cancer tissue

	Percentage conversion			
		[7-^{3}H]T		
Patient	[1β-^{3}H]A ^3H_2O	E_1	E_2	mRNA
1	0.184	—	—	None
2	0.271	0	0	None
3	0.178	0	0	—
4	0.224	0	0	—
5	0.227	0	0	—
6	0.092	0	0	None
7	0.069	0	0	—

Homogenates (30 mg) of prostatic tissue from patients with prostatic cancer were incubated with an NADPH generating system for 2 h at 37°C. The value for the heat inactivated sample from each tissue (mean 0.093%) was subtracted from all ^3H_2O results. Estradiol (E_2) and estrone (E_1) were purified by HPLC and TLC. Aromatase mRNA was analyzed by amplification PCR.

estrone or estradiol (Table 1). The limit of detection of the assay was 0.001%. Even when 5 μM progesterone was included in the incubation to inhibit 5α-reduction of the substrate and maximize the amount available for the aromatase pathway, no estrogen could be detected. The ^3H_2O release appeared to be an NADPH-dependent enzymatic process as the amount of ^3H_2O produced by control tissue incubated with NADPH was more than four times the value for tissue either inactivated and/or incubated without cofactors. The possibility that estradiol or estrone produced during incubation was rapidly converted by the prostate to other metabolites was also investigated. However, when prostatic cancer tissue was incubated with [4-^{14}C]estradiol and [4-^{14}C]estrone, more than 85% of the radioactivity was accounted for as estradiol or estrone, suggesting that neither estrogen was metabolized in significant amounts.

Our findings are quite similar to those reported by Stone *et al.* [16] who also measured release of ^3H_2O from [1β-^{3}H]androstenedione and [1,2,6,7,^{3}H]androstenedione. However, it appears from our study that the ^3H_2O is not associated with aromatization to form estrogens. Although the cause of the loss of tritium from C-1β to produce ^3H_2O is unknown, it appears to be due to a metabolic process.

We have further attempted to gain evidence for the presence of aromatase in prostatic cancers by mRNA amplification with the polymerase chain reaction (PCR). In this method mRNA is extracted from the tissue, and reverse transcribed. The cDNA is then subjected to PCR using *Thermus aquaticus* DNA polymerase and two 24-mer oligonucleotide primers specific for aromatase. Under these conditions, only the aromatase sequence is amplified. The resulting PCR mixture was subjected to electrophoresis on polyacrylamide gels before and after restriction enzyme digestion to confirm the identity of the amplified product. This method has been used in our laboratory to detect aromatase expression in human ovary, human placenta and human breast tumors. However, there was no evidence of aromatase in the six prostatic tumors (three tumors analyzed from the first set of patients and three from the second, as indicated in Table 1).

The effect of 4-OHA on 5α-reductase

Both testosterone and androstenedione were mainly converted to 5α-reduced metabolites by the prostate. As previously reported [34], 5α-reductase activity was increased in BPH tissue. We noted significantly greater conversion of androstenedione to 5α-androstanedione, although conversion to DHT was only slightly increased in BPH compared to normal prostatic tissue. Formation of 5α-reduced metabolites in tissues from both cancer and BPH patients was inhibited by 4-OHA, although the compound appeared to be more effective in hyperplastic tissue. When tissue was incubated with 5 μM 4-OHA and 10^{-8}–10^{-5} M testosterone, the apparent K_i of 4-OHA on the 5α-reductase was determined to be 0.69 μM, while the apparent K_m was 0.19 μM. This value is similar to the apparent K_i of 4-OHA in BPH tissue reported by Zoppi *et al.* [35] and by Houston and Habib [36]. Inhibition of 5α-reductase in cancer tissue by 4-OHA has not been previously reported. Due to insufficient amounts of malignant tissue we were not able to determine the K_i, but the extent of inhibition suggests that it is rather less than in BPH. Inhibition of 5α-reductase by 5 μM 4-OHA was 74.5% compared to 98.3% with 4-MA. 4-OHA appears to be a competitive inhibitor of 5α-reductase [36] but clearly is less potent than it is for aromatase. The 17β-hydroxy-4-aza-4-methyl-19norandrost-5-en-3-one (L651190) as well as progesterone also inhibited 5α-reductase. However, other aromatase inhibitors, CGS 16949A or PED were without effect [33].

CONCLUSIONS

While there appears to be a role for 4-OHA and other aromatase inhibitors in the treatment of breast cancer, a rationale for their use in prostatic cancer is less clear. A mechanism of

action involving intraprostatic aromatase is unlikely. However, inhibition of peripheral aromatization might be of benefit to patients with prostatic cancer or BPH. Peripheral aromatization increases with age in both men and women. Others have reported that 4-OHA affects prostatic 17-hydroxysteroid dehydrogenase which could result in greater conversion of testosterone to androstenedione and 5α-androstanedione rather than to the more potent androgen, DHT [37]. In a recent study of patients with prostatic cancer, Davis *et al.* found no reduction in plasma DHT levels although estradiol levels were significantly reduced. While only four patients responded objectively to treatment, 28 of 49 patients experienced subjective remission and reduction in pain [38]. 4-OHA has weak androgenic activity. It remains to be determined whether non-steroidal inhibitors can be more effective. As in the case of breast cancer, sequential use of compounds with different modes of action may be of value in extending the disease free interval in prostatic cancer patients.

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