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Estrogen Synthesis in Human Breast Tumor and Its Inhibition by Testololactone and Bromoandrostenedione[1]

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Abstract

A total of 53 tumors have been examined for estrogen synthesis from androstenedione and assayed for estradiol receptors. It was found that of the 40 tumors that metabolized androstenedione to estrogens, 17 tumors were estradiol receptor negative and 23 tumors were estradiol receptor positive. Of the 13 tumors that did not synthesize estrogens, 7 tumors were receptor negative and 6 tumors were receptor positive. No correlation was found between the ability of the tumor to synthesize estrogens and the presence or absence of estradiol receptors. The inhibition of aromatase enzyme in human breast tumors by Δ^1-testololactone, testololactone, and 6α- and 6β-bromoandrostenedione was investigated. Estrone and estradiol synthesis from androstenedione was reduced in five tumor incubations by the presence of 0.2 mM Δ^1-testololactone and testololactone. 6α- and 6β-bromoandrostenedione (2.0 μM) were also shown to block estrogen synthesis in 5 tumors. Furthermore, Lineweaver-Burk plots revealed that all four compounds were competitive inhibitors of androstenedione aromatization. An apparent K_m of the aromatase enzyme for androstenedione of 0.08 μM and a V_{max} of 23 pmol of estrone synthesized per g tumor per hr were determined for one human breast tumor specimen. The use of an aromatase inhibitor such as Δ^1-testololactone in the treatment of breast cancer should be reconsidered. Data from one patient with advanced cancer of the breast, responding to previous oophorectomy and adrenalectomy and treated with large doses of Δ^1-testololactone, are presented to illustrate the significance of successful treatment by scientific approaches.

Introduction

It has now been established that conversion of circulating androgens to estrogens is an ongoing process throughout the life of a woman. Several human tissues, including adipose tissue (12, 14, 16, 18, 20), liver (24), and muscle (13), have been shown to be possible sites for the peripheral aromatization of androgens. In women with breast cancer, the tumor itself has been demonstrated to be a source of local estrogen production (2, 11, 15, 17, 25). The pathophysiological significance of estrogen synthesis by tumor in women with breast cancer has yet to be elucidated. Similarly, the role of peripheral conversion of circulating androgens to estrogen in women with hormone-dependent mammary cancer must be investigated in order to elucidate the biochemical mechanism of growth regulation of these tumors.

Numerous aromatase inhibitors have been studied in human placental tissues. It has been demonstrated by Bellino *et al.* (5) that both 6α- and 6β-bromoandrostenedione are effective aromatase inhibitors in human placental microsomes. Δ^1-Testololactone, a drug which has been used with some success in the treatment of metastatic breast cancer, is also effective in the inhibition of aromatase in human placenta (22). Earlier, we reported that both 6α- and 6β-bromoandrostenedione, Δ^1-testololactone, and testololactone can inhibit the formation of estrogens from androstenedione by human mammary carcinoma *in vitro* (17). In this paper, we present data on an extended study of these aromatase inhibitors *in vitro* and the use of the aromatase inhibitor in the treatment of a woman with metastastic breast cancer with a relapse after a previous response to oophorectomy and adrenalectomy.

Materials and Methods

Chemicals. [7α-^{3}H]androstenedione (specific activity, 11 Ci/mmol), [4-^{14}C]estrone (55 mCi/mmol), [4-^{14}C]estradiol (55 mCi/mmol), and [4-^{14}C]estriol (55 mCi/mmol) were purchased from Amersham Searle Corporation, Arlington Heights, Ill., and [2,4,6,7-^{3}H]estradiol (100 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. The purity of these chemicals was checked on TLC.[2] Standard steroids were purchased from Steraloids, Wilton, N. H. All solvents were redistilled. Heptane was treated with H_2SO_4 and washed with alkaline $KMnO_4$ before distillation. The resin used for the ion-exchange column was Bio-Rad AG1-X2, 200 to 400 mesh, in the chloride form. The inhibitors Δ^1-testololactone and testololactone were kindly supplied by Dr. Albert Segaloff, Alton Ochsner Medical Foundation. 6α- and 6β-bromoandrostenedione were given by Dr. Yoshio Osawa, Buffalo Medical Foundation, Buffalo, N. Y.

Incubation, Isolation, and Identification of Estrogens. Tumors removed at the time of surgery were promptly trimmed of fat and connective tissues and frozen in liquid nitrogen. The specimens were later weighed and pulverized, and a portion (300 mg) was removed for estrogen receptor assay. Approximately 1.0 g was homogenized in 4 ml of 0.25 M sucrose and 1 ml of 0.5 M Tris buffer (pH 7.6) by a ground-glass homogenizer with a motor-driven pestle. To the homogenate were added an NADPH-generating system and 10 μCi [7α-^{3}H]androstenedione with and without inhibitors. The mixture was incubated for 3 hr at 37° in a Dubnoff metabolic shaking incubator. The reaction was stopped with 10 ml of hot acetone:ethanol (20:3), and the mixture was filtered through Whatman No. 1 paper. To the filtrate were added 8000 dpm [4-^{14}C]estrone, 4000 dpm [4-^{14}C]estradiol, 3000 dpm [4-^{14}C]estriol, and 500 μg of each of the 3 estrogens. After the organic solvents were evaporated, the radioactive metabolites were isolated by the method of Ryan and Smith (19). The steroids were extracted with chloroform and partitioned between heptane and 90% methanol. The methanol extract was run on an ion-exchange column. The neutral steroids were eluted with 25 ml methanol, and the phenolic steroids were eluted with 35 ml 80% methanol. The eluent was collected in

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[2] The abbreviation used is: TLC, thin-layer chromatography.

5-ml fractions. Tubes containing ^{14}C were combined and rerun on a second column to remove the contaminating neutral steroids. The phenolic fraction was run on TLC in ethylene glycol monomethyl ether:benzene (8:92) to separate the 3 phenolic estrogens. Approximately 12 mg carrier steroid were added, and the estrogens were recrystallized to constant specific activity. The final precipitate was acetylated, and the acetates were run on TLC in ethyl acetate:cyclohexane (30:70). If sufficient material remained, the acetates were recrystallized. Aliquots of the previous samples were counted in duplicates in 10 ml liquid scintillation fluid to S.E. <5% on a Beckman LS-250 liquid scintillation counter with automatic quench compensation.

Estrogen Receptor Assay. Quantitative determination of estradiol receptor was carried out by a method described previously (21). The tumor cytosol was incubated with 5 nM [2,4,6,7-^3H]estradiol for 4 hr at 0° in the presence and absence of nafoxidine. A dextran-coated charcoal pellet was used to remove the excess steroid. The samples were layered on top of 10 to 30% sucrose gradients and centrifuged for 16 hr at 50,000 rpm with a SW60 rotor in a Beckman Model L5-50 centrifuge. The pmol of estradiol bound per mg of protein were determined in the 4S and 8S region of the gradient.

Results

Relationship between Tumor Estradiol Receptors and Aromatase Activity. The data for identification of the estrogen metabolites of androstenedione formed by breast tumors from a total of 13 tumors were presented in a previous paper (25). The 3 estrogen fractions from TLC were purified until the ^3H:^{14}C ratio was constant or fell below 1.0. Carrier recrystallization and derivative formation gave positive identification of these metabolites. No evidence for estriol formation was found in any of these tumors.

In the earlier study of these tumors, 7 tumors were estrogen receptor negative and 6 tumors had aromatase activity. Ten tumors were estrogen receptor positive, and 7 of these tumors also showed the presence of aromatase activity. These results showed a lack of correlation between estrogen synthesis by these tumors and the presence or absence of estrogen receptors. The results of the extended study of a total of 53 tumors are summarized in Chart 1, which shows clearly a lack of correlation between the ability of the tumors to synthesize estrogens and the presence or absence of estradiol receptors in these tumors.

Kinetic Analysis of Aromatase and its Inhibitors. The study of the time course of the aromatase reaction was reported in an earlier publication (6). The data disclosed that the amount of estrogen synthesized from androstenedione increased linearly throughout the first 40 min of the reaction and then reached a plateau. Thus, the 30-min incubation time was chosen for the kinetic studies. The kinetic analysis of aromatase and its inhibitors, Δ^1-testololactone and testololactone, was studied and reported in a paper published previously (6). The incubation was carried out with concentrations of [7α-^3H]androstenedione ranging from 0.05 to 0.8 μM. The results of this experiment were reported in pmol of estrone, since 90% of the estrogen formed by this tumor was estrone. A reciprocal plot of the velocity of the reaction *versus* the substrate concentration revealed an apparent K_m of the aromatase enzyme for androstenedione of 0.08 μM and a V_{max} of 23 pmol of estrone synthesized per g tumor per hr.

Inhibition of Aromatase by Δ^1-Testololactone and Testololactone. A total of 5 tumors, all synthesizing estrogens from androstenedione, were studied for inhibition of aromatization by testololactone. All tumors were incubated with 0.4 μM [^3H]androstenedione in the presence and absence of 0.2 mM Δ^1-testololactone or testololactone. The aromatase enzyme was inhibited in all 5 tumors and the range of inhibition was 75 to 88% by Δ^1-testololactone and 50 to 80% by testololactone. Table 1 summarizes the results of inhibition of aromatase enzyme in 2 tumors by these inhibitors. The data suggest that Δ^1-testololactone is a better aromatase inhibitor. A Lineweaver-Burk plot (Chart 2) shows the convergence of all 3 lines at the *ordinate* and is indicative of competitive inhibition of aromatase enzymes by these compounds.

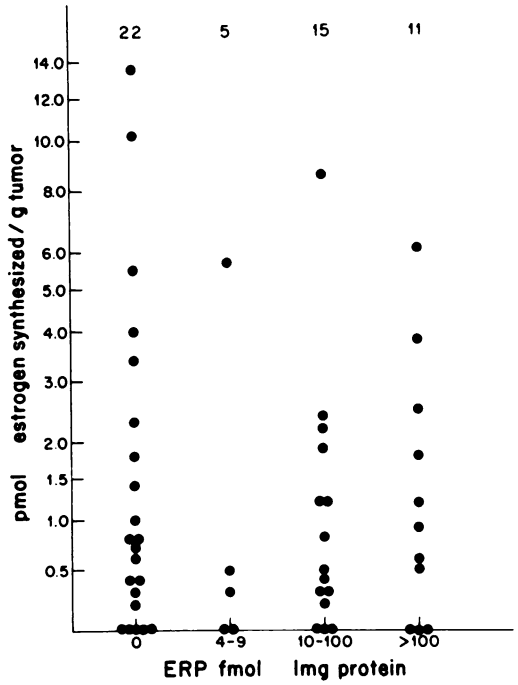

Chart 1. Estrogen synthesis compared with ERP.

Inhibition of Aromatase by 6α- and 6β-Bromoandrostenedione. A total of 5 tumors, all synthesizing estrogens from androstenedione, were examined for inhibition of aromatase by 6α- and 6β-bromoandrostenedione. The tumors were incubated with 0.4 μM [^3H]androstenedione in the presence and absence of 2.0 μM 6α- or 6β-bromoandrostenedione. Aromatization of androstenedione was inhibited in all 5 tumors, and the range of inhibition was 73 to 95% for 6α-bromoandrostenedione and 73 to 82% for 6β-bromoandrostenedione. Table 2 gives the data on inhibition of aromatase in 2 tumors by these 2 inhibitors. It appears that 6α-bromoandrostenedione is a more effective aromatase inhibitor than is its 6β isomer. Again, the Lineweaver-Burk plot (Chart 3) discloses that these compounds are competitive inhibitors of this enzyme.

Effect of Aromatase Inhibitor in a Patient with Metastatic Breast Cancer. The following is an illustration of the use of Teslac in a woman with metastatic breast cancer, who responded previously to oophorectomy and adrenalectomy. She is now being treated with Teslac, following evidence of progression of her disease. The patient is a 53-year-old woman, who, prior to 1968, had approximately 10 biopsies for recurrent breast lesions. This led to bilateral s.c. mastectomy with implants, which was performed in May 1968. In May 1973, she

developed bilateral carcinoma of the breast, Stage IV, and underwent bilateral oophorectomy. Objective regression was observed until August 1974, when she developed multiple skin and s.c. metastases. Bilateral adrenalectomy was performed in December 1974, and objective regression was observed until April 1979. At that time, increased skin lesions were present. These lesions were excised, and estrogen receptor assay was carried out, revealing an estrogen binding level of of 64.0 fmol/mg protein. In April 1980, the patient developed increased skin metastasis with pleural effusion; estrogen receptor assay was again performed following excision of these lesions and was 74.4 fmol/mg protein. Plasma estradiol was measured at this time and was found to be 80 pg/ml; plasma estrone was not detected. Treatment was begun with Δ^1-testololactone (Teslac), 1000 mg p.o., daily. In November 1980, the skin lesions were regressing. She had gained 6 kg, and her blood pressure was slightly higher. Plasma estrogens at this time were not detectable. The dosage of Teslac was reduced to 800 mg/day. The patient was seen again 6 months later in May 1981, at which time the lesions were continuing to regress slowly. Estrogen receptor binding was 48 fmol/mg protein. The patient was continued on Teslac, 800 mg/day. In November 1981, the lesions were still regressing, and the patient's weight and blood pressure had returned to initial pretreatment levels. The patient continues on treatment with Teslac, 800 mg/day.

Table 1

Inhibition of aromatase by Δ^1-testololactone and testololactone

Tumors were incubated for 3 hr with 0.4 μM [7α-^{3}H]androstenedione with or without 0.2 mM Δ^1-testololactone and testololactone.

Patient		pmol/g tumor E_1	pmol/g tumor E_2	% of inhibition
M. F.	No inhibitor	0.0	1.6	
	Δ^1-Testololactone	0.2	0.2	83
	Testololactone	0.4	0.4	67
P. W.	No inhibitor	19.7	1.0	
	Δ^1-Testololactone	2.2	0.2	88
	Testololactone	3.9	0.2	80

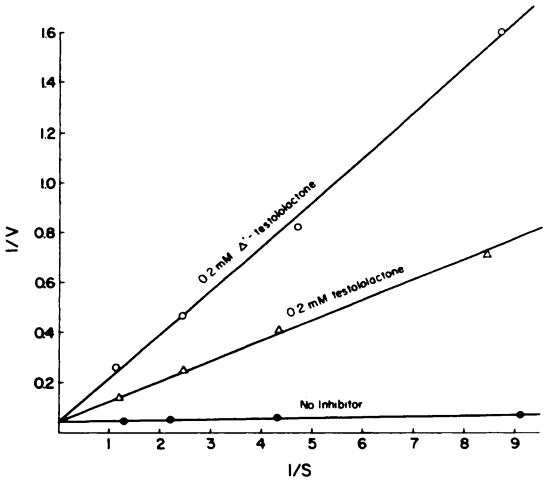

Chart 2. Inhibition of estrogen synthesis from androstenedione in human mammary tumor by testololactone. *V*, pmol estrone synthesized per g tumor per hr; *S*, μM androstenedione; ●, no inhibitor; ○, 0.2 mM Δ^1-testololactone; △, 0.2 mM testololactone.

Table 2

Inhibition of aromatase by 6α- and 6β-bromoandrostenedione

Tumors were incubated for 3 hr with 0.4 μM [7α-^{3}H]androstenedione with or without 2.0 μM 6α- and 6β-bromoandrostenedione.

Patient		pmol/g tumor E_1	pmol/g tumor E_2	% of inhibition
P. W.	No inhibitor	20.4	0.9	
	6α-Bromoandrostenedione	1.4	0.1	93
	6β-Bromoandrostenedione	3.7	0.1	82
A. F.	No inhibitor	1.5	1.9	
	6α-Bromoandrostenedione	0.2	0.2	88
	6β-Bromoandrostenedione	0.4	0.3	73

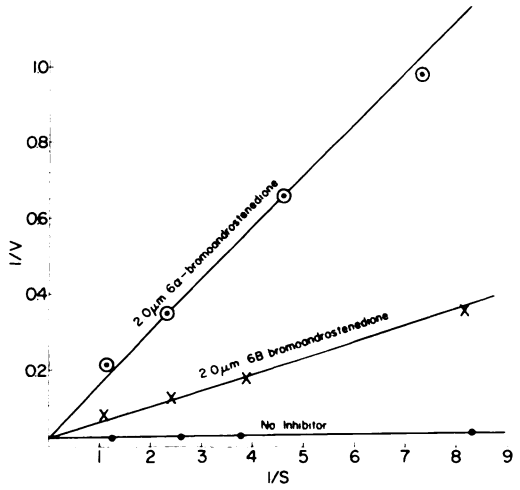

Chart 3. Inhibition of estrogen synthesis from androstenedione in human mammary tumor by bromoandrostenedione. *V*, pmol estrone synthesized per g tumor per hr; *S*, μM androstenedione; ●, no inhibitor; ⊙, 2.0 μM 6α-bromoandrostenedione; ×, 2.0 μM 6β-bromoandrostenedione.

Discussion

The isolation of 11β-hydroxy estrogens in the urine of oophorectomized and adrenalectomized women with breast cancer led to the postulation by Chang and Dao (7) that the tumor might be the site of aromatization. This postulate was supported by the subsequent observation of these authors that human breast tumor slices could convert cortisone to 11β-hydroxy estrogens (8). Since then, there have been many reports of metabolic conversion of testosterone (3) and dehydroepiandrosterone (1) to estradiol, androstenedione to estrone (23), and cholesterol to androgens (10) by human breast tumors.

Altogether, these studies demonstrate conclusively that many human breast tumors are capable of synthesizing estrogens by metabolic conversion of steroid precursors. The fact that there is a lack of correlation between the ability of breast tumors to synthesize estrogen and the presence or absence of estrogen receptors in these tumors suggests that this is not a biochemical property of hormone-independent neoplasia.

The clinical implications of tumors synthesizing estrogens are of interest and of practical importance. In the treatment of hormone-dependent breast cancer, endocrine-ablative procedures are effective presumably because of the removal of the source of estrogens or precursor steroids (9). The ability of peripheral tissues to convert precursor steroids to estrogens,

even in the absence of gonads and adrenals, raises the important question whether the continued presence of estrogens as a result of the peripheral metabolism contributes to the tumor growth after a period of remission. In these patients, further treatment with agents capable of inhibiting estrogen synthesis can be expected to offer continued control of the disease.

In the present study, we found that Δ^1-testololactone, testolactone, and 6α- and 6β-androstenedione are competitive inhibitors of aromatase in human breast tumors. Δ^1-Testololactone was found to be more effective at reducing the level of estrogen than was testololactone. 6α-Bromoandrostenedione was more potent than its 6β isomer, and this parallels the results of Bellino *et al.* (5) using human placenta. Our data show that 6α-bromoandrostenedione was the most effective aromatase inhibitor of the 4 compounds studied.

Although inhibition of peripheral metabolism of androstenedione by Δ^1-testololactone in breast cancer patients was reported by Barone *et al.* (4), our *in vitro* studies demonstrate that estrogen formation can be blocked directly at the tumor site. Furthermore, Barone *et al.* found that only the level of plasma estrone was reduced in the presence of Δ^1-testololactone. We have shown also that the concentration of estradiol was lowered.

Since breast tumor is well established as one site of peripheral formation of estrogens and we have shown that aromatase inhibitors can block this synthesis, such compounds could be of therapeutic value in breast cancer. It is of particular interest to note the continued presence of estrogen receptors in tumors of ovariectomized and adrenalectomized women and the ability of these tumors to synthesize estrogens. These biochemical properties are indicative of the persistance of hormone dependency in these tumors. Such patients are candidates for treatment with aromatase inhibitors.

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