Report

The effects of aromatase inhibitors and antiestrogens in the nude mouse model

Qing Lu, Wei Yue, Jiping Wang, Yang Liu, Brian Long, and Angela Brodie
Department of Pharmacology & Experimental Therapeutics, and Greenbaum Cancer Center, School of Medicine, University of Maryland, Baltimore, Maryland, USA

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Summary

The effects of antiestrogens, tamoxifen and ICI 182,780, and aromatase inhibitors, arimidex (anastrozole ZD1033) and letrozole (CGS 20,267), on the growth of tumors were studied in nude mice. In this model, estrogen dependent MCF-7 human breast cancer cells stably transfected with the aromatase gene were inoculated in four sites per mouse. Sufficient estrogen is produced from aromatization of androstenedione supplement (0.1 mg/mouse/day) by the cells to stimulate their proliferation, tumor formation, and maintain the uterus similar to that of the intact mouse. Once the tumors reached a measurable size, the mice were injected with antiestrogen or inhibitor for 35–56 days. Tumor volumes were measured weekly. At autopsy, the tumors were removed, cleaned, and weighed. Statistical data was determined from tumor weights. Both antiestrogens were effective in reducing tumor growth in these mice. Tamoxifen appears to be more effective than ICI 182,780, although the former stimulated the uterine weight whereas the pure antiestrogen did not. However, both aromatase inhibitors were more effective than the antiestrogens. Tumor regression was observed with letrozole. Thus, after-treatment tumor weights were less than those of a group of mice at the start of treatment. The aromatase inhibitors also reduced the weight of the uterus, suggesting that these compounds, as well as the pure antiestrogen, may not cause endometrial proliferation, unlike tamoxifen. These aromatase inhibitors may not only benefit patients who have relapsed from tamoxifen, but may be more effective in patients as first line agents for suppressing the effects of estrogen.

Introduction

Breast cancer is the most common malignant neoplasm in U.S. women. Although causes of breast cancer are not well understood, the contribution of estrogens to the development of the normal breast and the growth of breast cancer has been widely documented [1, 2]. Approximately one-third of human breast cancers are estrogen dependent. The dependence of breast tumors on estrogens increases with age. Thus, tumors of postmenopausal patients are more likely to be dependent on estrogens for their progression than those of younger women. Estrogen receptor positive tumors are generally more differentiated, slower growing, and indicate a more positive prognosis for the patient. Estrogens are synthesized by aromatization of androgen substrates via a series of reactions catalyzed by cyto-

Address for offprints and correspondence: A. Brodie, Department of Pharmacology & Experimental Therapeutics, 655, W. Baltimore St., School of Medicine, University of Maryland, Baltimore, MD 21201, USA; *Tel:* 410-706-3137; *Fax:* 410-706-0032; *E-mail:* abrodie@umaryland.edu

chrome P450 aromatase (P-450arom). Following menopause, when the ovary is no longer the main source of estrogen, production is increased in peripheral tissue, such as adipose and muscle which make up most of the body mass. Thus, systemic approaches to treatment rather than surgical removal of adrenals or the pituitary, are proving more effective, well tolerated, and associated with less morbidity and mortality. For patients who have estrogen and/or progesterone receptor positive tumors, endocrine therapies are more successful than chemotherapy [3].The effect of estrogens on the growth of breast cancers can be blocked by two ma nipulations: inhibition of estrogen action by antiestrogens, which interact with estrogen receptors [4], and blockade of estrogen synthesis by inhibitors of aromatase [5].

Tamoxifen (ICI 46,474), a nonsteroidal antiestrogen, was developed in 1967 and entered clinical trials for advanced breast cancer in 1971 [6]. Its use as an adjuvant to surgery and as the first line therapy for advanced disease in postmenopausal patients is now established [3]. Tamoxifen competes with estrogen for binding to the estrogen receptor, and blocks the action of estrogen [4]. Adjuvant tamoxifen therapy for early breast cancer (both node positive and node negative) produces reduction in annual rates of recurrence and death (25% for recurrence and 17% for mortality). Tamoxifen also reduced the risk of development of contralateral breast cancer by 39% [7]. However, tamoxifen is a weak or partial agonist and for this reason it may not be the optimal agent for inhibiting hormone dependent tumor growth. It has also been reported that tamoxifen treatment increases the risk of developing endometrial carcinoma, and the incidence is correlated with the duration of treatment [8]. This effect is thought to be due to the partial agonistic action of tamoxifen which in rodents produces a uterotrophic response [9].

In the late 1980's, steroidal antiestrogens were developed (10–13) which are more potent than tamoxifen as antiestrogens and are without agonistic effects. In patients with breast cancer, faslodex (ICI 182,780: 7α-(9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl]estra-1,3,5(10)-triene-3,17β-diol) reduces ER content in the tumors and the concentration of two estrogen regulated proteins, progesterone receptor and pS2 [13]. Clinical trials with ICI 182,780 in breast cancer patients are in progress [14].

In the early 1970's, our laboratory reported the first of a series of compounds which selectively inhibit estrogen synthesis [15]. A number of steroidal agents were found to be potent aromatase inhibitors and subsequently 4-hydroxyandrostenedione was shown to be effective in treating breast cancer [16–19]. Recently, several non-steroidal selective inhibitors have been developed which are imidazole and triazole derivatives based on antifungal agents that inhibit P-450 enzymes. Two triazole compounds have recently been approved as second line agents in the treatment of advanced breast cancer in postmenopausal patients. They are arimidex (ZD1033:2,2'[5-(1*H*-1,2,4-triazol-1-yl methyl)-1,3--phenylene]bis(2-methylpropiononitrile) and letrozole (CGS 20267: 4-[1-(cyanophenyl)-1-(1,2,4-triazolyl)methyl]benzonitrile). Both are very potent and selective aromatase inhibitors and are well tolerated by patients. Letrozole (CGS 20267) is approximately 100 times more potent than fadrozole (CGS 16949A, an imidazole analogue) in reducing serum estradiol levels, which may be due to the significantly longer half-life of the former in patients [20]. Doses of 1 to 10 mg arimidex have been reported to reduce plasma estradiol levels to the limit of the detection assay in postmenopausal breast cancer patients [21]. Recent clinical studies that compared these inhibitors to other second line agents, found that letrozole caused more complete and partial tumor responses than aminoglutethimide [22] and that arimidex increased survival of patients to a significantly greater extent than megace [23]. However, the optimal use of these well-tolerated agents remains to be determined.

Because of its established efficacy, most patients receive tamoxifen as a first line agent. Thus, direct comparison with new agents is a difficult and slow process. We, therefore, established a model for postmenopausal, hormone-dependent, breast cancer in nude mice in which antiestrogens and aromatase inhibitors could be compared [24]. In this model, estrogen receptor positive human breast cancer cells (MCF-7) transfected with the aromatase gene are inoculated into ovariectomized nude mice.

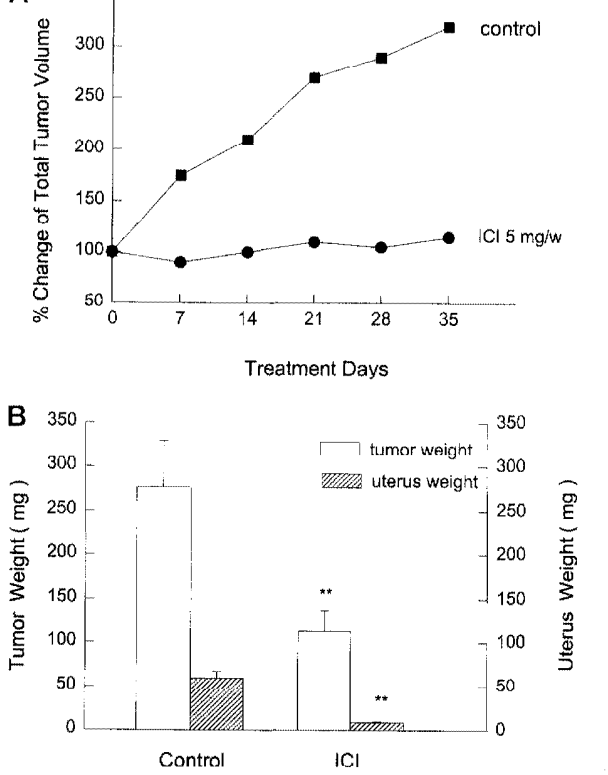

Figure 1. The effect of antiestrogen, ICI 182,780 on tumor and uterine wet weight in the nude mouse model. Groups of 4 mice with tumors of MCF-7 breast cancer cells transfected with the aromatase gene were injected sc with the antiestrogen, ICI 182,780, 5 mg/mouse/week, or vehicle. A. Tumors were measured weekly and the percentage change in volume calculated. B. After 35 days of treatment, mice were sacrificed, tumors and uteri were weighed. Values, mean ± SE, are significantly different from control ** $p < 0.01$.

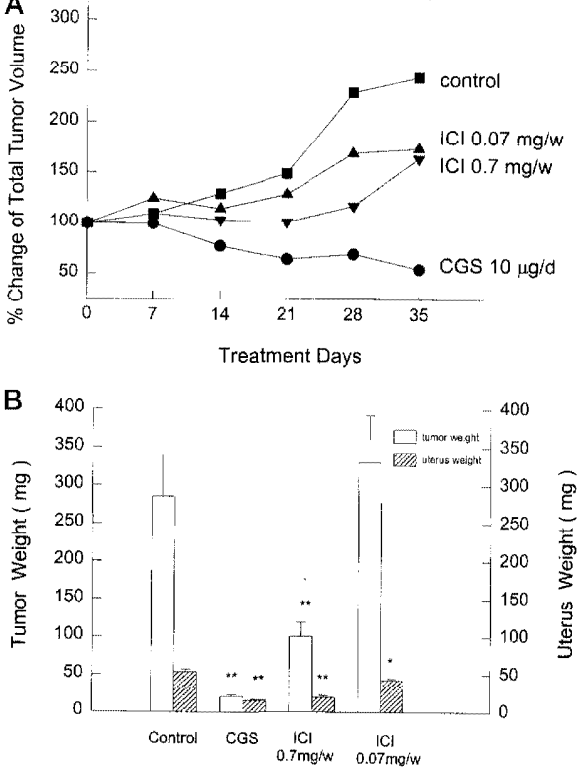

Figure 2. The effect of antiestrogen ICI 182,780 and aromatase inhibitor, letrozole on tumor and uterine wet weight in the nude mouse model. Groups of 4 mice were injected sc with ICI 0.7 mg or 0.07 mg in oil once per week, with letrozole (CGS) 10 µg in 0.3% HPC/mouse/day, or with 0.3% HPC vehicle. A. Tumors were measured weekly and the percentage change in volume calculated. B. After 35 days of treatment, mice were sacrificed, and tumors and uteri were weighed. Values, mean ± SE, are significantly different from control * $p < 0.05$; ** $p < 0.01$.

These cells synthesize endogenous estrogen which is sufficient to stimulate their proliferation into tumors and maintain the uterus in these animals. In the present study, we have used this model to investigate the effects of the aromatase inhibitors letrozole and anastrozole, and the steroidal antiestrogens faslodex (ICI 182,780) and tamoxifen, on estrogen target tissues, mammary tumors and the uterus.

Materials and methods

Athymic mice

Female BALB/c athymic mice 4-6 weeks of age (20–22 g body weight) were obtained from NCI, Frederick, MD. The animals were housed in a pathogen-free environment under control led conditions of light and humidity and received food and water *ad libitum*. Ovariectomy was carried out under fluorothane anesthesia 1–3 days before cell inoculation.

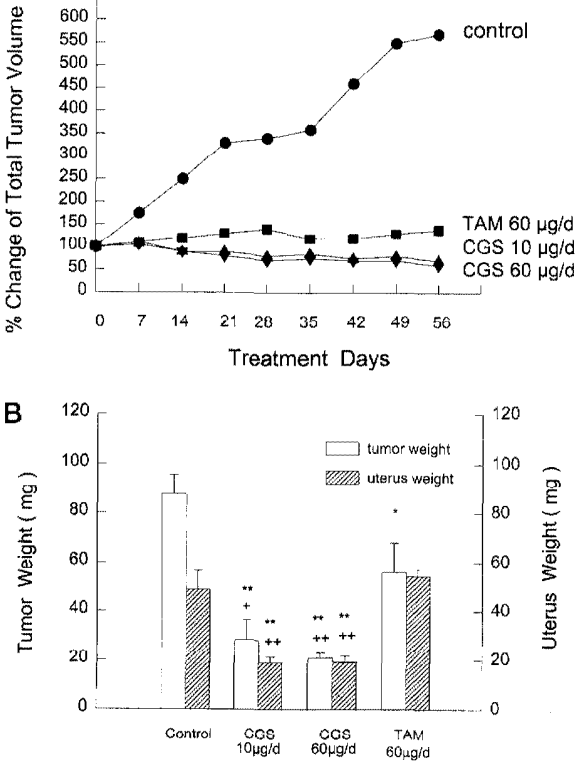

Figure 3. The effect of antiestrogen tamoxifen and aromatase inhibitor letrozole on tumor and uterine wet weight in the nude mouse model. Groups of 4 mice were injected sc daily with letrozole (CGS) 10 µg/mouse/day or 60 µg/mouse/day, tamoxifen 60 µg/mouse/day, or vehicle. A. Tumors were measured weekly and the percentage change in volume calculated. B. After 56 days of treatment, mice were sacrificed, and tumors and uteri were weighed. Values, mean ± SE, are significantly different from control * $p < 0.05$, ** $p < 0.01$; CGS 10 µg vs. TAM * $p < 0.05$; CGS 60 µg vs. TAM + $p < 0.01$.

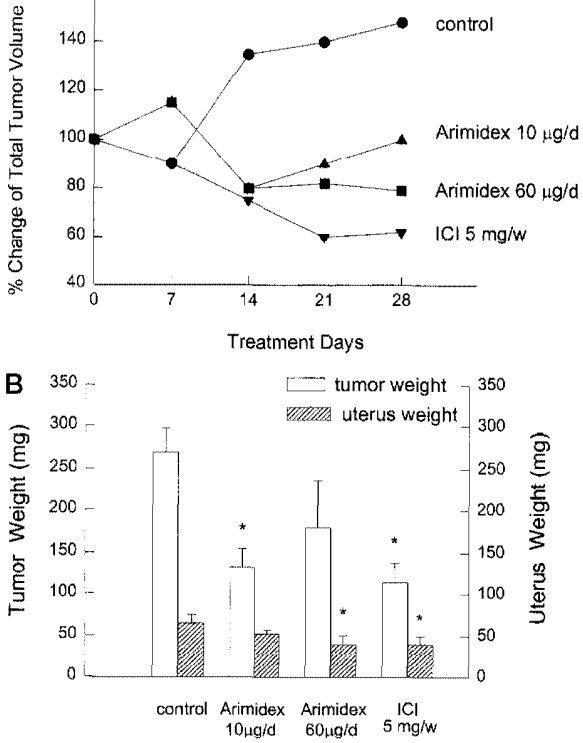

Figure 4. The effect of antiestrogen ICI 182,780 and aromatase inhibitor arimidex on tumor and uterine wet weight in the nude mouse model. Groups of 4 mice were injected sc daily with arimidex 10µg/mouse/day or 60 µg/mouse/day or ICI 182,780 (ICI) 5mg/week sc or vehicle. A. Tumors were measured weekly and the percentage change in volume calculated. B. After 28 days of treatment, mice were sacrificed, and tumors and uteri were weighed. Values, mean ± SE, are significantly different from control * $p < 0.05$.

Table 1. The effect of letrozole and tamoxifen on tumor weight

Treatment	Treatment Days	Mice n	Tumors n	Tumor mg wet weight[a]
vehicle	1	4	21	[b] 53.54 ± 7.51
vehicle	56	2	17	226.3 ± 31.10
tamoxifen	56	4	26	[b] 55.88 ± 12.20
letrozole	56	4	22	[b] 20.58 ± 2.09
letrozole[c]	35-56	2	14	[b] 74.64 ± 9.10

Groups of four mice were injected sc with tamoxifen or letrozole (60 µg/mouse/day) or vehicle. One group of vehicle-treated mice were autopsied on Day 1, and tumors were removed and weighed. All other mice were autopsied on Day 56 of treatment. [a] Mean ± SE; [b] values are significantly different from control ($p < 0.01$). [c] Two mice in the control group were crossed over to letrozole treatment on Day 21.

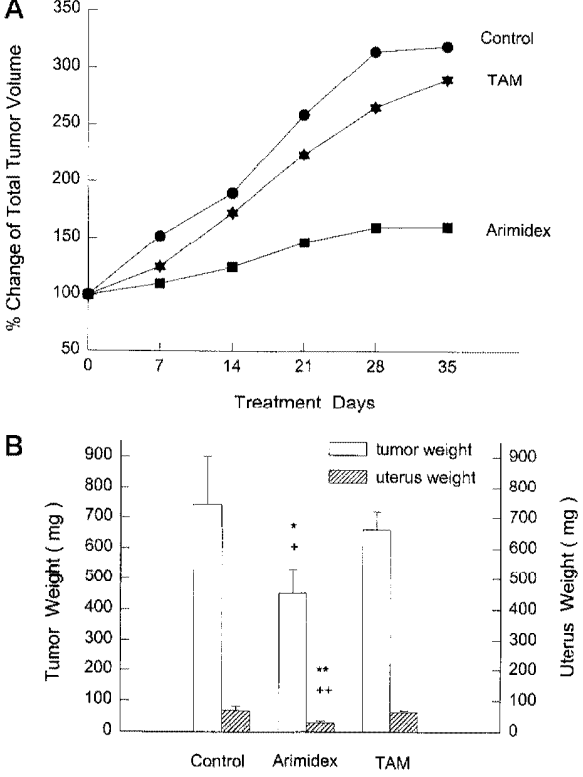

Figure 5. The effect of antiestrogen tamoxifen and aromatase inhibitor arimidex on tumor and uterine weight in the nude mouse model. Groups of 5 mice were injected sc daily with tamoxifen 3µg/mouse/day or arimidex 5µg/mouse/day or vehicle. A. Tumors were measured weekly and the percentage change in volume calculated. B. After 35 days of treatment, mice were sacrificed, tumors and uteri were weighed. Values, mean ± SE, are significantly different from control * $p < 0.05$; ** $p < 0.01$; arimidex vs. TAM $^{-}$ $p < 0.05$; $^{+}$ $p < 0.01$.

Cell culture and inoculation to athymic mice

As reported previously [24], we used MCF-7 cells stably transfected with the human placental aromatase gene (MCF-7_{CA}). The cells were cultured in Eagle's minimum essential medium containing 5% fetal bovine serum and neomycin (600 µg/ml; GIBCO, Bethesda, MD). The culture medium was changed twice weekly. Subconfluent MCF-7_{CA} cells were scraped into Hank's solution and centrifuged at 1,000 rpm for 2 min at 4° C. The cells were then resuspended in Matrigel (10 mg/ml) (kindly provided by Dr. Hynda Kleinman, NCI) to make a cell suspension of 2–5 10^7 cells/ml. Each mouse was inoculated sc in four sites with 0.1 ml of the cell suspension. Animals were then injected sc daily with 0.1 mg androstenedione/mouse. Growth rates were determined by measuring the tumors with calipers every week. Tumor volumes were calculated according to the formula $4/3\times\pi\times r_1^2\times r_2$ ($r_1<r_2$).

Treatment

Treatment began 21 to 35 days after androstenedione injections when tumors had reached a measurable size. Mice were treated daily with sc injections of aromatase inhibitors, letrozole (CGS 20,267) (kindly provided by Dr. Ajay Bhatnagar, Novartis, Basel Switzerland) and arimidex (kindly provided by Dr. Michael Dukes, Zeneca Pharmaceuticals, Macclesfield, UK) or the antiestrogen tamoxifen in 0.3% hydroxypropylcellulose (HPC). The antiestrogen ICI 182,780 was injected in oil once per week (kindly provided by Dr. A Wakeling, Zeneca Pharmaceuticals, Macclesfield, UK). Control animals were given injections of vehicle (0.3% HPC, 0.1 ml/mouse/day) sc daily. The treatment lasted 4–5 weeks. Animals were autopsied 4 hours after the last injection. The uteri were removed, cleaned, and weighed. Multiple small tumors were removed from each inoculation site and their total weight determined. All compounds were very well tolerated and no adverse effects were observed.

Statistics

One way ANOVA was used to analyze the data.

Results

1. ICI 182,780

Treatment of a group of four mice with either ICI 182,780 (5 mg/mouse/week; i.e., 35.7 mg/kg/week, sc) or vehicle was started 28 days after inoculation. There was no significant change in tumor volume in the ICI treated mice (789.99 ± 65.24 mm^3 to 889.07 ± 155.45 mm^3) during the 35 days of treatment. However, tumor volume in the control mice increased from 635.82 ± 57.15 mm^3 to

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