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Report

The effect of combining aromatase inhibitors with antiestrogens on tumor growth in a nude mouse model for breast cancer

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Summary

We have previously established a model for postmenopausal, hormone-dependent breast cancer in nude mice which is responsive to both antiestrogens and aromatase inhibitors. In this model, MCF-7 human breast carcinoma cells transfected with the aromatase gene (MCF-7_{CA}) synthesize sufficient estrogen to form tumors in ovariectomized nude mice. In the present study we used this intratumoral aromatase model to investigate the effects on tumor growth of the new nonsteroidal aromatase inhibitors letrozole (CGS 20,267) and anastrozole (ZD 1033) and the antiestrogens tamoxifen (ICI 47,474) and faslodex (ICI 182,780). Furthermore, we determined whether the inhibition of estrogen synthesis together with inhibition of estrogen action would be more effective in controlling breast tumor growth. The results of our studies indicate that the aromatase inhibitors anastrozole and letrozole, as well as the new pure antiestrogen faslodex, have potent antitumor effects in the mouse model. In the treatment of mice with mammary tumors, letrozole was more effective in suppressing tumor growth than anastrozole. This was consistent with the Ki values of these inhibitors against placental aromatase and the IC_{50} values in cell culture (MCF-7_{CA}), which indicated the greater potency of letrozole as an aromatase inhibitor. Letrozole also had greater antitumor effects than tamoxifen and faslodex. The antitumor effect of letrozole was substantial, making it difficult to detect any additional effect on the tumors when letrozole was combined with the antiestrogens. However, the combined treatment of anastrozole + tamoxifen and anastrozole + faslodex also did not increase efficacy compared to the aromatase inhibitor alone. In addition, combining the two antiestrogens did not suppress tumor growth more effectively than faslodex alone. Our results show that treatment with the combinations of aromatase inhibitors with either tamoxifen or faslodex are not more effective in blocking estrogen stimulation of tumor growth than the aromatase inhibitors alone.

Abbreviations: Faslodex (ICI 182,780): 7α-[9-(4,4,5,5,5-pentafluoropentylsulfinyl) nonyl]estra-1,3,5(10)-triene-3,17β-diol; Letrozole (CGS 20,267): 4-[1-(cyanophenyl)-1-(1,2,4-triazolyl)methyl]benzonitrile; Anastrozole (ZD 1033): 2,2′[5-(1H-1,2,4-triazol-1-yl-methyl)-1,3-phenylene] bis(2-methlypropiononitrile); HPC: hydroxypropyl cellulose.

Introduction

Estrogens are known to be important in the growth of breast cancers in both pre and postmenopausal women. Estrogen receptor concentrations in breast cancers increase with age even after menopause. This results in a higher fraction of postmenopausal patients with hormone sensitive cancers than among premenopausal patients [1]. While the ovary is no longer the major source of estrogens in older women, production is increased in peripheral sites and contributes to the stimulation of breast cancers [2].

Two strategies that are now used to ameliorate the growth effects of estrogens are inhibition of estrogen action by compounds interacting with estrogen receptors, and inhibition of estrogen synthesis. The first method used clinically was the inhibition of estrogen action. The antiestrogen tamoxifen (ICI 46,474), a

nonsteroidal antiestrogen, was developed in 1967 and entered clinical trials for advanced breast cancer in 1971 [3]. Tamoxifen is effective as adjuvant therapy for postmenopausal, hormone responsive breast cancer [4]. However, it has been reported that long-term tamoxifen treatment increases the risk of developing endometrial carcinoma and the incidence is correlated with the duration of treatment [5]. This effect is thought to be due to the partial agonistic action of tamoxifen. The steroidal antiestrogen faslodex (ICI 182,780) was developed in the late 1980's. This compound is a more potent antiestrogen than tamoxifen and is without agonistic effects [6, 7]. Clinical trials with faslodex in breast cancer patients are in progress.

Estrogen synthesis is mediated by the enzyme aromatase and is the last step in the steroidogenic pathway. Inhibition of aromatase should effectively reduce estrogen production from all sources in the body without affecting the biosynthesis of other steroid hormones. Selective aromatase inhibitors were first reported by our laboratory in 1973 [8]. One of these compounds, 4-hydroxyandrostenedione [9], has now been developed for breast cancer treatment [10, 11]. Recently, several non-steroidal aromatase inhibitor compounds have been reported [12]. These include triazole derivatives based on antifungal agents that inhibit P-450 enzymes. Triazoles are noted for their favorable pharmacology. Two triazole compounds, letrozole (CGS 20,267) [13] and anastrozole (ZD 1033) [14], are now approved as second line agents in the treatment of advanced breast cancer in postmenopausal patients. Both are selective aromatase inhibitors and are well tolerated by patients [15–18].

Comparison of these new agents is difficult in the clinic since patients receive approved drugs until they relapse. Therefore, most studies to date with aromatase inhibitors and new antiestrogens have been carried out in patients with advanced disease who have been previously treated with tamoxifen or other agents. To address this problem, we developed a model for postmenopausal breast cancer which is responsive to both antiestrogens and aromatase inhibitors [19, 20]. After menopause, estrogens are produced by the aromatization of androgens in non-ovarian tissue [1], including the normal breast [21] and breast tumors themselves [21–23]. In our model, hormone responsive MCF-7 human breast cancer cells are transfected with the human aromatase gene and serve as the source of estrogen in the ovariectomized mouse. In the present study, we used this intratumoral aromatase model to determine the effects on tumor growth of the new nonsteroidal aromatase inhibitors letrozole and anastrozole, and the antiestrogens tamoxifen and faslodex. Furthermore, we addressed the question of whether the inhibition of estrogen synthesis together with inhibition of estrogen action would be more effective in controlling breast tumor growth than either one alone. Therefore, the effect of combined treatment with aromatase inhibitors and antiestrogens was also investigated.

Methods

Radiometric (^3H_2O) assay of aromatase in human placental microsomes

Preparation of human placental microsomes and aromatase activity assays were performed as previously described [9,19]. Briefly, 200 μg of placental microsomes were mixed with 0.3 μCi of [1β-^{3}H]-androstenedione, androstenedione (0–2000 nM), with/without 5 μM of the aromatase inhibitors letrozole and anastrozole, and 1.25 IU/ml of the NADPH generating system (NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) in 1 ml 0.1 M phosphate buffer, pH 7.4. Following a 30 min incubation at 37°C, the assay was terminated by adding 2 ml of chloroform to extract unconverted substrate and other steroids. An aliquot of 0.7 ml of the aqueous phase was treated with 2.5% activated charcoal suspension (0.7 ml) to remove any residual steroids. Tritiated water (^3H_2O) formed during the aromatization of [1β-^{3}H]-androstenedione to estrogen was measured by counting the radioactivity in the aqueous supernatant. The Ki values were determined by the Lineweaver and Burke method.

Radiometric (^3H_2O) assay of aromatase in MCF-7_{CA} cells

Cell Culture

Human breast carcinoma cells (MCF-7) stably transfected with the human placental aromatase gene (MCF-7_{CA}) were used as previously described [19]. The cells were cultured in Eagle's minimum essential medium containing 5% fetal bovine serum and neomycin (600 μg/ml). The culture medium was changed twice weekly.

Assay

MCF-7_{CA} cells were plated into 6-well plates (100,000 cells/well) and left overnight to attach. For

determination of IC_{50} values for the aromatase inhibitors, the cells were washed and incubated for 2 h with 0.5 μCi of [1β^{3}H]-androstenedione in 1 ml of medium in the presence of letrozole or anastrozole at concentrations of 0.001–100 nM. After incubation, medium was transferred to a glass test-tube and 300 μl of trichloracetic acid was added to precipitate the proteins. After centrifugation, 1 ml of medium was mixed with 2 ml of chloroform to extract unconverted substrate and other steroids. The aromatization assay was then performed as above [19].

Athymic mice

Female BALB/c athymic mice 4–6 weeks of age were obtained from Charles River Laboratories (Boston, MA). The animals were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water *ad libitum*. Ovariectomy was carried out under metathane anesthesia 1–3 days before cell inoculation.

Inoculation of MCF-7$_{CA}$ cells to athymic mice

Subconfluent MCF-7_{CA} cells were scraped into Hank's solution and centrifuged at $1000 \times g$ for 10 min at 4°C. The cells were then resuspended in Matrigel (10 mg/ml, kindly provided by Dr Hynda Kleinman, NIH) to make a cell suspension of 3×10^7 cells/ml. Each mouse was inoculated sc in four sites with 0.1 ml of the cell suspension. Beginning on the day of inoculation and for the duration of the experiment, animals were injected with 0.1 mg per mouse per day sc androstenedione, substrate for aromatization to estrogens. Animals were weighed and tumor growth rates were determined by measuring the tumors with calipers every week. Tumor volumes were calculated according to the formula $4/3 \times \pi \times r_1^2 \times r_2 (r_1 < r_2)$ [19, 20].

Treatments

Treatments began when all tumors had reached a measurable size (~500 mm^3). This occurred 28–35 days after androstenedione injections began. Mice were then treated daily with sc injections of the aromatase inhibitors, letrozole (CGS 20,267; MW 285.1) (kindly provided by Dr Ajay Bhatnagar, Novartis, Basel Switzerland) and anastrozole (ZD 1033; MW 293.4) (kindly provided by Dr Michael Dukes, Zeneca Pharmaceuticals, Macclesfield, UK), and with tamoxifen (ICI 47,474), or a combination of letrozole plus tamoxifen, or anastrozole plus tamoxifen in 0.3% hydroxypropylcellulose (HPC). The antiestrogen faslodex (ICI 182,780) was injected once per week in oil (as kindly provided by Dr. A Wakeling, Zeneca Pharmaceuticals, Macclesfield, UK). Control animals were given injections of the vehicle (0.3% HPC, 0.1 ml/mouse/day) sc daily. The treatments lasted 5–6 weeks as indicated in the figures. Animals were autopsied 4–6 h after the last injection. Tumors and uteri were removed from the mice, cleaned, and weighed.

Tumor estradiol and letrozole concentrations

Tumors from mice in each treatment group were pooled and homogenized in PBS buffer at 4°C. The steroids were extracted with diethyl ether and estradiol isolated by celite chromatography. Estradiol concentrations were measured in triplicate aliquots of the homogenates by RIA. The assay was performed using an estradiol antibody and iodinated estradiol (ICN, Boston). The sensitivity of the assay was 1 pg/ml [25, 26]. Serum concentrations of letrozole were measured using HPLC/UV and kindly performed by G. Leferre, Novartis Pharma, DMPK, France.

Statistics

The effects of treatment on tumor weights were compared using one way ANOVA followed by Newman Keuls multiple range test when required.

Results

Ki values for the aromatase inhibitors letrozole and anastrozole were obtained by incubating placental microsomes with 0.3 μCi [1β^{3}H]-androstenedione for 30 min with a range of inhibitor concentrations. The Ki value was 1.02×10^{-9} M for letrozole and 5.35×10^{-9} M for anastrozole. Thus, letrozole is approximately five times more potent than anastrozole in inhibiting aromatase activity of placental microsomes. Both compounds showed competitive inhibition of the enzymes, as previously reported [13, 14].

Inhibition of aromatase was determined in semi-confluent cultures of MCF-7_{CA} cells. IC_{50} values for the aromatase inhibitors letrozole and anastrozole were obtained by incubating MCF_{CA} cells with 0.5 μCi [β^{3}H]-androstenedione for 2 h in the presence of compounds. The experiment was repeated three times and the mean values were presented in Figure 1. The IC_{50} value was 0.35 ± 0.07 nM (SE) for letrozole

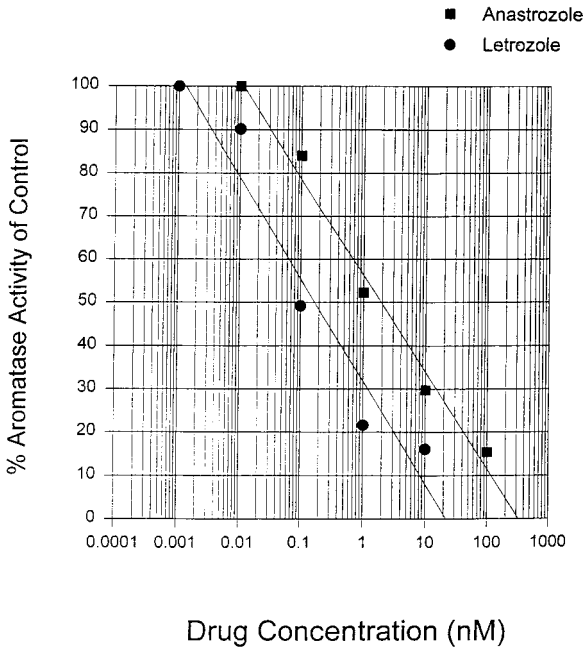

Figure 1. The IC_{50} values for letrozole (CGS 20,267) and anastrozole (ZD 1033) in human breast carcinoma cells transfected with the aromatase gene (MCF-7_{CA}). Semi-confluent cultures of cells were incubated with 0.5 μCi [1β-^{3}H]-androstenedione for 2 h in the presence of the concentrations of drugs indicated.

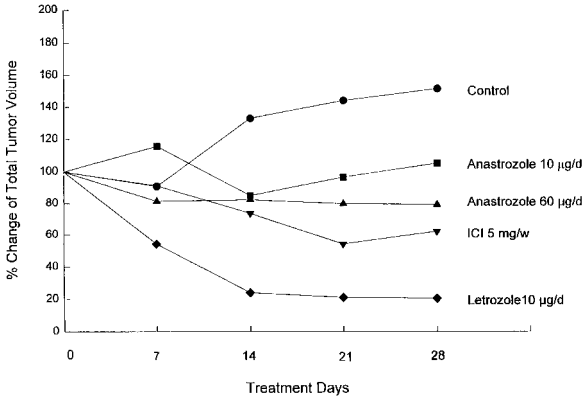

Figure 2a. Comparison of the effect of doses of anastrozole (10 and 60 μg per mouse per day), faslodex (ICI, 5 mg per mouse per week), and letrozole (10 μg per mouse per day) on the volume of MCF-7_{CA} breast tumors in ovariectomized nude mice.

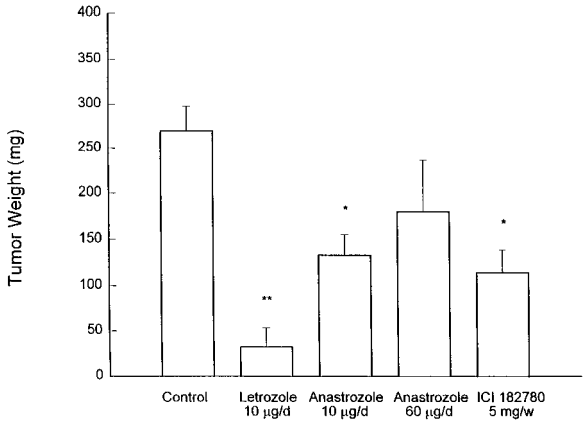

Figure 2b. Comparison of the effect of treatment with anastrozole (10 and 60 μg per mouse per day), faslodex (ICI, 5 mg per mouse per week), and letrozole (10 μg per mouse per day) for 28 days on the mean tumor weights of ovariectomized nude mice.

and 3.62 ± 0.90 nM (SE) for anastrozole. Letrozole is approximately 10-fold more potent than anastrozole in inhibiting aromatase activity in the MCF_{CA} cells.

Several doses of antiestrogens and aromatase inhibitors were initially evaluated (Figures 2A & 2B). Twenty-eight days after inoculation, groups of five mice received letrozole (10 μg per mouse per day, sc), anastrozole (10 μg per mouse per day, sc), anastrozole (60 μg per mouse per day, sc), or faslodex (5 mg per mouse per week, sc). After treatment for 28 days, the total tumor volumes for control mice had increased by 145.9% (Figure 2A.). The total tumor volumes in the anastrozole (10 μg), anastrozole (60 μg per mouse per day, sc), and faslodex groups decreased and to about the same extent during treatment, to 95.6%, 78.2%, and 63.2%, respectively. Tumor volume was markedly decreased by letrozole treatment to 22.4%. The mean tumor weights for letrozole (32.1 ± 21.0 mg), anastrozole (10 μg per mouse per day, 133.2 ± 22.0 mg), and faslodex (114.9 ± 24.4 mg) were significantly less than for the controls (270.0 ± 27.7 mg) ($p < 0.05$) (Figure 2B). However, tumor weights of the anastrozole (10 and 60 μg) treated mice were not significantly different from each other or from faslodex treated animals. Although anastrozole (10 μg/day) showed an erratic pattern of growth and regression during the few weeks of treatment, the other treatments showed more typical gradual reduction in tumor volume.

Since the experiment in Figure 2A indicated that 10 μg per mouse per day letrozole caused marked tumor regression, 5 μg per mouse per day was initially evaluated along with tamoxifen (3 μg per mouse per day) (Table 1, # 1). In this experiment, the mean tumor weight for mice treated for 42 days with letrozole (5 μg per mouse per day) was 30.7 ± 5.2 mg, with tamoxifen (3 μg per mouse per day) was 128.7 ± 20.1 mg, and with letrozole plus tamoxifen was 115.5 ± 19.4 mg. All values were significantly less than the controls (166.7 ± 32.1 mg) ($p < 0.05$). In addition, the mean tumor weight of the letrozole group

Table 1. The effect of aromatase inhibitors and antiestrogens on tumor and uterine weight

Experiment	Treatment	n	t	Tumor weight (mg)	Uterine weight (mg)
1	Control	5	42	146.7 ± 32.1	43.5 ± 10.1
	Letrozole			$30.7 \pm 5.2^{a,b}$	26.2 ± 4.2^{a}
	Tamoxifen			128.7 ± 20.1	50.6 ± 7.6
	Let + Tam			115.5 ± 19.4	51.0 ± 3.9
2	Control	5	35	743.3 ± 157.4	68.3 ± 14.0
	Anastrozole			452.9 ± 78.9^{a}	28.0 ± 7.2^{a}
	Tamoxifen			661.9 ± 58.3	62.8 ± 4.5
	Anast + Tam			412.7 ± 90.2^{a}	48.0 ± 5.0
3	Control	4	42	297.6 ± 54.5	45.6 ± 2.4
	Letrozole			22.9 ± 3.7^{a}	$20.2 \pm 1.1^{a,b}$
	Tamoxifen			178.9 ± 22.9^{a}	60.7 ± 3.2
	Let + Tam			$52.0 \pm 8.9^{a,b}$	$41.2 \pm 2.4^{a,b}$
	Anastrozole			$104.3 \pm 14.3^{a,b}$	24.5 ± 1.2^{a}
	Anast + Tam			$132.8 \pm 26.5^{a,b}$	44.6 ± 2.2
4	Control	5	35	506.8 ± 112.5	46.7 ± 3.3
	Letrozole			$42.5 \pm 6.8^{a,b,c,d}$	19.4 ± 0.9
	Faslodex			182.0 ± 35.5^{a}	14.6 ± 2.1^{a}
	Let + Fas			$66.2 \pm 16.9^{a,b,c,d}$	14.5 ± 1.2^{a}
	Anastrozole			$137.4 \pm 19.3^{a,b}$	18.4 ± 2.7^{a}
	Anast + Fas			220.7 ± 27.8^{a}	19.8 ± 4.5^{a}
	Tamoxifen			255.0 ± 55.1	32.5 ± 2.5
	Tam + Fas			345.4 ± 56.6	24.8 ± 4.9

Groups of ovariectomized nude mice each with four tumors of MCF-7 breast cancer cells transfected with the aromatase gene were injected sc daily with androstenedione (0.1 ml per mouse per day). When tumors had reached a measurable size, the mice were treated with vehicle (controls), letrozole (let: 5 μg per mouse per day sc), anastrozole (anast: 5 μg per mouse per day sc), tamoxifen (tam: 3 μg per mouse per day sc), or faslodex (fas: 70 μg per mouse per week sc). Animals were sacrificed on the last day of treatment and tumors and uteri removed and weighed.
n = number of mice.
t = duration of treatment.
[a] $p < 0.05$ from control.
[b] $p < 0.05$ from tamoxifen.
[c] $p < 0.05$ from faslodex.
[d] $p < 0.05$ from anastrozole.

was significantly less than of the tamoxifen and the letrozole plus tamoxifen groups ($p < 0.05$). Tamoxifen treatment resulted in a reduction in tumor growth, whereas with letrozole treatment, tumor weights actually decreased from initial volumes. This experiment determined that letrozole was effective at 5 μg per mouse per day for 42 days. In this model, uterine weight is maintained by estrogen produced by the tumor [20, 26]. The mean uterine wet weight of the group treated with letrozole (26.2 ± 4.3 mg) was significantly less than the control group (43.5 ± 10.1 mg) ($p < 0.05$) (Table 1). In contrast, the mean uterine weight of the animals treated with letrozole plus tamoxifen was not significantly different from those of mice treated with tamoxifen alone or vehicle. The concentration of letrozole measured in pooled serum was 90 nmol/l for the group treated with letrozole alone, and 74.3 nmol/l for the group treated with letrozole plus tamoxifen; none was detected in the serum of the control group.

In the second experiment (Table 1, # 2), we compared the effects of anastrozole (5 μg per mouse per day) in combination with tamoxifen. Treatments were started in groups of five mice, 35 days after inocu-

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