Effect of the Selective Estrogen Receptor Modulator Arzoxifene on Repopulation of Hormone-Responsive Breast Cancer Xenografts between Courses of Chemotherapy

Licun Wu and Ian F. Tannock

Abstract

Selective inhibition of repopulation of clonogenic tumor cells between courses of chemotherapy has potential to improve the effectiveness of treatment. Here we study arzoxifene, a short-acting selective estrogen receptor modulator, for its potential to inhibit repopulation in estrogen-dependent human breast cancer MCF-7 xenografts between courses of chemotherapy. Proliferation of tumor cells was evaluated by cyclin D1 expression and uptake of 5-bromo-2'-deoxyuridine. Arzoxifene decreased cell proliferation in xenografts. To model adjuvant treatment of human breast cancer, MCF-7 cells were injected s.c. into nude mice and four groups of mice received the following treatments beginning after implantation: (a) control (vehicle solution); (b) arzoxifene alone, 5 days per week by oral gavage for 3 weeks; (c) 5-fluorouracil (5-FU) or paclitaxel i.p. weekly, for 3 doses; and (d) arzoxifene following each cycle of chemotherapy. The incidence of tumors with volume ≥50 mm³ was determined as a function of time. MCF-7 xenografts developed in 100% of control mice by 4 weeks after implantation. Paclitaxel or 5-FU alone had minor effects to delay the appearance of xenografts whereas arzoxifene alone caused longer delay. Combined treatment with arzoxifene given between cycles of 5-FU or paclitaxel had substantial effects, with ~50% tumor incidence by 5 weeks. Our results indicate that arzoxifene can inhibit repopulation of hormone-responsive MCF-7 breast cancer xenografts when given between courses of chemotherapy. The scheduling of short-acting hormonal agents between courses of adjuvant chemotherapy for human breast cancer has potential to improve the outcome of treatment.

The long-term survival of patients with hormone-responsive breast cancer can be improved by adjuvant treatment using both chemotherapy and hormonal agents such as tamoxifen (1). A large clinical trial has compared concurrent use of chemotherapy and tamoxifen with sequential treatment in which tamoxifen was initiated following 3 to 6 months of chemotherapy (2). Sequential treatment was superior, presumably because tamoxifen may render the tumor cells noncycling so that they become more resistant to cycle-active chemotherapy. However, sequential treatment also has disadvantages because it delays administration of an effective therapy (tamoxifen) and proliferation of surviving cancer cells may occur between treatments with chemotherapy. This repopulation may limit the net cell killing due to chemotherapy (3, 4). The administration of a short-acting hormonal agent between cycles of chemotherapy might inhibit repopulation, but allow retention of sensitivity to cycle-active drugs, if it is stopped before the next cycle of chemotherapy.

Repopulation has been studied most often during radiotherapy where it has been shown to have a substantial effect on the outcome of treatment. Moreover, the repopulation of tumor cells during daily-fractionated irradiation may accelerate with time (5, 6). Repopulation is likely to be even more important during the longer intervals between courses of chemotherapy (3). We have shown that the rate of repopulation in mouse breast tumors between weekly courses of treatment with cyclophosphamide or 5-fluorouracil (5-FU) may also increase with time (7). Repopulation of surviving tumor cells during courses of chemotherapy is an important and rather neglected mechanism that can lead to effective drug resistance and limit the outcome of treatment (3, 4).

Strategies that attempt to alter tumor proliferation kinetics by using tumor-selective cytostatic agents might improve therapeutic index by limiting tumor cell repopulation during courses of chemotherapy. One approach is to use selective estrogen receptor modulators as inhibitors of repopulation of hormone-sensitive breast cancers. Arzoxifene is a novel, third-generation selective estrogen receptor modulator with a much shorter half-life (~30 hours) than tamoxifen (~7 days; refs. 8, 9). We have shown that arzoxifene and 4-hydroxy-tamoxifen are effective in inhibiting the growth of estrogen receptor-positive human breast cancer cell lines MCF-7 and T47D in tissue culture and inhibit the repopulation of these cells during courses of chemotherapy with 5-FU or methotrexate (10). Here, we study the inhibitory effects of arzoxifene on growth and cell proliferation.
of established estrogen receptor–positive breast cancer xenografts and its effect to delay or prevent the appearance of xenografts when given to mice soon after transplantation of breast cancer cells. We compare the use of chemotherapy alone, arzoxifene alone, or arzoxifene given between courses of chemotherapy to inhibit repopulation of tumor cells, thus modeling strategies that might be used as adjuvant therapy in patients.

Materials and Methods

Cell lines and confirmation of estrogen receptor-α status by immunohistochemical staining. Most experiments were done using estrogen receptor–positive MCF-7 cells, which were maintained in α-MEM supplemented with antibiotics and 10% FCS. The cells were provided by American Type Culture Collection (Manassas, VA) and confirmed Mycoplasma-free. MDA-231 cells, maintained in a similar way, were used as a control when determining estrogen receptor-α status of tumors by immunostaining as previously described (10).

Drug preparation and delivery. Arzoxifene (also known as LY353381.HCl) was provided by Lilly Corporate Center (Indianapolis, IN). Arzoxifene was first dissolved in DMSO (Fisher Chemicals, Fair Lawn, NJ) and then diluted with 0.5% methylcellulose (Sigma, St. Louis, MO) in water. The final concentration of DMSO was <0.01%. Arzoxifene was administered daily by oral gavage in a total volume of 0.5 mL/mouse. 5-FU (Pharmacia & Upjohn, Inc., Mississauga, ON) or paclitaxel (Bristol-Myers Canada, Inc., Montreal, Canada) was obtained from the hospital pharmacy and was injected i.p. once weekly.

Generation of xenografts. Female, 4- to 5-week-old athymic nude mice, purchased from Harlan Sprague-Dawley Laboratory Animal Center (Madison, WI) were acclimatized in the animal colony for 1 week before experimentation and were housed in microisolation cages, five per cage, in a 12-hour light/dark cycle. Filtered sterilized water and sterile rodent food were given ad libitum. Just before transplantation of estrogen receptor–positive MCF-7 (but not ER-MDA-231) cells, a 0.72-mg 17-estradiol 60-day release pellet (E2 pellet; Innovative Research of America, Sarasota, FL) was implanted s.c. in the bilateral auxiliary mammary fat pads of female Harlan Sprague-Dawley nude mice, and 4 hours later, the animals were divided into four groups and treatments were initiated according to the schedule shown in the figure.

**Immunohistochemistry.** Paraffin sections were dewaxed in five changes of xylene and passed through graded alcohols. Endogenous peroxidase and biotin activities were blocked using 3% hydrogen peroxide and Vector's avidin/biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA), respectively. Sections were treated with 0.25% pepsin for 5 minutes at room temperature for BrdUrd staining (7). The primary antibody, human anti-BrdUrd (clone II-4, Caltag, Burlingame, CA; 1:1,000), was incubated for 1 hour. The other sections were treated with microwave heating in 10 mmol/L citrate buffer at pH 6.0 inside a pressure cooker to enhance the
sensitivity to an antibody against cyclin D1 (13). They were incubated overnight in a moist chamber with the primary antibody, mouse anti-human monoclonal cyclin D1 (clone DCS-6, DAKO, Santa Cruz, CA), at a dilution of 1:50. Sections were rinsed in PBS thrice for 5 minutes. This was followed by 30 minutes each with biotinylated horse anti-mouse immunoglobulin G (Vector) and horseradish peroxidase–conjugated streptavidin (Ulta HRP Detection System, ID Labs, Inc., London, ON, Canada). Color development was undertaken with freshly prepared Nova Red solution (Vector) and sections were counterstained with Mayer’s hematoxylin. Finally, sections were dehydrated through graded alcohols, cleared in xylene, and mounted in Permount (Fisher).

Quantification of cell proliferation. Sections were imaged using a CoolSnap Pro color video camera mounted on a Nikon OPTIPHOT microscope (Japan). At least 10 fields (200×) were randomly selected from each section and the positively and negatively stained tumor cell nuclear areas were collected by using Image-Pro Plus 5.0 analysis software (Media Cybernetics, Silver Spring, MD). BrdUrd labeling indices were determined as the ratio of areas occupied by the positively stained tumor cell nuclei to all tumor cell nuclei (7). Necrotic areas were excluded. Cyclin D1 staining was quantified in a similar way. The relative area of cyclin D1 staining was calculated based on the ratio of positively stained cell nuclei to all tumor cell nuclei (7). Necrotic areas were excluded. Cyclin D1 staining was quantified in a similar way. The relative area of cyclin D1 staining was calculated based on the ratio of positively stained cell nuclei to all tumor cell nuclei (7). Necrotic areas were excluded. Cyclin D1 staining was quantified in a similar way.

Confirmation of estrogen receptor-α status of xenografts
MCF-7 cells stained strongly positive for estrogen receptor-α although the staining was heterogeneous (Fig. 2A). MDA-231 cells were included as a negative control.

Effect of arzoxifene on cell proliferation
Cyclin D1 expression. Expression of cyclin D1 after treatment with vehicle solution or arzoxifene is shown in Fig. 2B. Cyclin D1 expression was unevenly distributed and positively stained cells tended to be located at the periphery of the tumor. Treatment of mice with arzoxifene resulted in a rapid decrease in cyclin D1 expression by day 7, which persisted throughout the period of treatment (Figs. 2B and 3A). However, on day 28 (i.e., 10 days after completing treatment), cyclin D1 expression increased to a level similar to that in the controls (Fig. 3A).

5-Bromo-2'-deoxyuridine incorporation. BrdUrd immunostaining after treatment with either vehicle solution or arzoxifene is shown in Fig. 2C. The labeling indices at different times are shown in Fig. 3B. Treatment with arzoxifene resulted in a decrease in the BrdUrd labeling index compared with the controls (P < 0.001 for each comparison on days 7, 14, and 21). However, the BrdUrd index recovered to control levels on day 28 (i.e., 10 days after the end of treatment).

Effect of arzoxifene on growth of established tumors
Growth of MCF-7 tumor was evaluated during and after daily oral administration of arzoxifene or vehicle solution (Fig. 4). Tumors grew relatively slowly, with a doubling time of ~47 days in controls. Arzoxifene caused marked inhibition of tumor growth (Fig. 4).
Generation of MCF-7 xenografts after treatment

The time-dependent incidence of xenografts with volume \( \geq 50 \text{ mm}^3 \) after various treatments is shown in Figs. 5A and 6A. MCF-7 xenografts grew to \( \geq 50 \text{ mm}^3 \) in 100% of control mice at \( 4 \) weeks after tumor cell implantation and estrogen supplementation. Treatment with 5-FU alone (Fig. 5A) or paclitaxel alone (Fig. 6A) had minimal and nonsignificant effects to delay the appearance of xenografts and there were greater and significant effects from administration of arzoxifene alone \([P < 0.0001 \text{ (Fig 5A)} \text{ and } P = 0.0005 \text{ (Fig. 6A)}]\). The development of MCF-7 xenografts in the groups treated with arzoxifene following 5-FU or paclitaxel was slower than in those treated with vehicle solution \((P < 0.0001 \text{ for each comparison})\), those treated with chemotherapy alone \((P < 0.0001 \text{ for both comparisons})\), and those treated with arzoxifene alone \([P = 0.035 \text{ (Fig. 5A)} \text{ and } P = 0.27 \text{ (Fig. 6A)}]\).

The number of tumors that grew to a size \( \geq 50 \text{ mm}^3 \) in each group by the end of the experiment on day 52 is indicated in Figs. 5B and 6B, as well as the median volume of tumors \( \geq 50 \text{ mm}^3 \). It is evident that combined treatment using arzoxifene following 5-FU or paclitaxel has substantial and significant effects to decrease tumor incidence and tumor volume at this time.

Toxicity of arzoxifene to mice

All animals were active during the whole period of the experiments (\(~ 60 \) days). Body weight of mice did not change significantly during and after 3 weeks of treatment with arzoxifene and/or chemotherapy.

Discussion

Considerable evidence has shown that hormonal therapy, either alone or in combination with chemotherapy, provides clinical benefit for patients with breast cancer that expresses estrogen receptors. However, there are few studies which have investigated the use of hormonal agents to inhibit the repopulation of surviving tumor cells between courses of chemotherapy. Our previous in vitro data show that repopulation of estrogen receptor–positive breast cancer cells MCF-7 and T47D between courses of chemotherapy was specifically inhibited by the selective estrogen receptor modulators arzoxifene and 4-hydroxy-tamoxifen whereas there was no effect on estrogen receptor–negative MDA-231 cells \((10)\). In the present study, we found that growth of established breast cancer xenografts derived from estrogen receptor-\(\alpha\)-positive MCF-7 cells was inhibited by arzoxifene although we found no evidence of shrinkage of the tumors as might be expected if the agent were directly toxic to the cells.

Our data indicate that the proliferation of MCF-7 cells in xenografts, as indicated by uptake of BrdUrd, was reduced after treatment with arzoxifene, given 5 days per week by oral gavage. The expression of cyclin D1 also decreased. Studies of invasive breast cancer have found that high levels of cyclin D1 are associated with estrogen receptor positivity \((14, 15)\) and that estrogen can induce cyclin D1 gene expression in estrogen receptor–positive human breast cancer cell lines \((16)\). Our observations indicate that the antiestrogen arzoxifene can...
reduce cyclin D1 expression with an associated decrease in cell proliferation. This inhibition is temporary because on day 28, 10 days after completing treatment with arzoxifene, BrdUrd uptake of BrdUrd at weekly time points after treatment with arzoxifene. It has been reported that arzoxifene resulted in a significant increase in estrogen receptor expression and a reduction in progesterone receptor expression whereas changes in cyclin D1 score were inversely related to p27 (kip1) score (17, 18).

The slow growth of MCF-7 xenografts and the limited life span of mice make it difficult to evaluate combined treatment when using established xenografts. Instead, we have attempted to model the treatment of microscopic disease, as in adjuvant chemotherapy following surgery for breast cancer in women. In mice implanted with MCF-7 tumor cells and with an estrogen-release pellet, there was 100% tumor growth and we were able to determine the effects of various treatments on the rate of development of palpable tumors. Chemotherapy alone, using either 5-FU or paclitaxel, had only small effects to delay the appearance of palpable xenografts and there were slightly greater effects due to arzoxifene alone. The doses of chemotherapy selected were similar to those used by others (19), but lower than the maximal tolerated doses (20), because we wanted to evaluate repopulation of surviving cells. Although higher doses of chemotherapy might be feasible, toxicity would have certainly occurred with repeat dosing and intermittent use of arzoxifene (with the daily handling of animals over long periods that this entails). When used in combination, arzoxifene was stopped 2 days before the next course of chemotherapy to allow the cells to reenter cell cycle (although a longer interval might have been optimal) because chemotherapeutic drugs are selective for cycling cells. Combined treatment had effects to delay or prevent the appearance of xenografts when arzoxifene was given between courses of chemotherapy, an effect that was probably due to inhibition of repopulation between cycles of chemotherapy. The strategy provides a model of alternating therapy with chemotherapy and short-acting hormonal agents, which is a promising approach to improve the effectiveness of combined therapy for estrogen-responsive breast cancer patients.

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