Therapeutic Strategies Using the Aromatase Inhibitor Letrozole and Tamoxifen in a Breast Cancer Model

Brian J. Long, Danijela Jelovac, Venkatesh Handratta, Apinya Thiantanawat, Nicol MacPherson, Joseph Ragaz, Olga G. Goloubeva, Angela M. Brodie

Background: The antiestrogen tamoxifen has potent activity against estrogen receptor–positive breast cancer, but two nonsteroidal aromatase inhibitors, letrozole and anastrozole, show considerable advantages over tamoxifen with respect to patient survival and tolerability. To determine the optimal way to use letrozole and tamoxifen, we studied their effects on a breast tumor xenograft model, MCF-7Ca, that is responsive to both antiestrogens and aromatase inhibitors. Methods: Female ovariectomized BALB/c athymic nude mice carrying xenograft tumors were treated daily subcutaneously with one of the following first-line therapies for varying durations: no drug (control), tamoxifen (100 µg/day) alone, letrozole (10 µg/day) alone, both drugs at the same time, or alternating 4-week courses of each drug (beginning with a course of tamoxifen or beginning with a course of letrozole). Tumor volumes and weights were estimated using linear mixed-effects models. The time to tumor doubling was calculated, and tumor weights in the treatment groups were compared, with adjustments for multiple comparisons being made with either Tukey’s or Dunnett’s procedure. Second-line therapies (with tamoxifen, letrozole, or fulvestrant) were initiated when tumors doubled in size under first-line therapies. All statistical tests were two-sided. Results: The times for doubling of tumor volume were as follows: control, 3–4 weeks; tamoxifen alone, 16 weeks; tamoxifen alternating with letrozole, 17–18 weeks; tamoxifen plus letrozole, 18 weeks; letrozole alternating with tamoxifen, 22 weeks; letrozole alone, 34 weeks. First-line treatment with letrozole was superior to treatment with tamoxifen alone or with the two drugs combined (at week 16, both \( P < 0.001 \)). Alternating tamoxifen and letrozole and alternating letrozole and tamoxifen were also not as effective as letrozole alone (at week 16, \( P = 0.002 \) and \( P < 0.001 \), respectively). Tumors progressing on tamoxifen remained sensitive to second-line therapy with letrozole compared with those remaining on tamoxifen at the end of treatment (week 28, \( P < 0.001 \)), whereas tumors progressing on letrozole were unaffected by second-line treatment with the antiestrogens tamoxifen or fulvestrant. Conclusions: First-line letrozole therapy extends time for tumor progression in this model relative to the other treatment regimens tested. However, further studies are needed to determine the most effective second-line therapy for tumors that progress on letrozole. [J Natl Cancer Inst 2004; 96:456–65]

The aromatase inhibitors anastrozole, letrozole, and exemestane are presently being compared with the antiestrogen tamoxifen as first-line treatment for postmenopausal patients with hormone-dependent advanced breast cancer (1–5) and as adjuvant therapies for patients with early-stage breast cancer (6,7). Results to date indicate that aromatase inhibitors are more effective and better tolerated than tamoxifen. Adjuvant tamoxifen has proved to be a remarkably successful breast cancer therapy (8,9). Tamoxifen blocks the action of estrogen at the receptor level, whereas aromatase inhibitors block the synthesis of estrogen in non-ovarian tissues, including breast tissue of postmenopausal patients. Consequently, there is considerable interest in determining the best strategy for using these agents to extend the time to disease progression and to increase patient survival rates. Although several trials are underway [reviewed in (7)], the clinical effects of combining and/or alternating aromatase inhibitors and tamoxifen have not been determined. As a guide for optimizing treatment of postmenopausal breast cancer patients, we developed a postmenopausal breast cancer model that is sensitive to both antiestrogens and aromatase inhibitors (10,11). In this model, hormone-responsive MCF-7 human breast cancer cells stably transfected with the human aromatase gene (MCF-7Ca) (12) are grown as tumors and serve as an autocrine source of estrogen in ovariectomized, immune-suppressed mice (10,11). In this regard, the model simulates the postmenopausal breast cancer patient, in whom the major source of the hormone is non-ovarian tissue, including normal and malignant breast tissue (13,14), and hormone production is not under gonadotropin regulation. Because the uterus is very sensitive to estrogen, change in uterine weight in these mice serves as a bioassay of endogenous estrogen levels. Because the production of adrenal androgens is deficient in these mice (15), we supplement them with Δ4-androstenedione. We have shown previously that Δ4-androstenedione has no direct effect on tumor growth (10). The aims of the study were to determine whether tumor progression in this model could be delayed by combining and/or alternating letrozole and tamoxifen therapies and whether tumors progressing on one type of therapy remained sensitive to second-line treatment with the other class of agent.

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See “Notes” following “References.”

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MATERIALS AND METHODS

Materials

MCF-7 human breast cancer cells stably transfected with the human aromatase gene (MCF-7Ca) were provided by Dr. S. Chen (City of Hope, Duarte, CA) (12). Dulbecco’s modified Eagle medium, penicillin (10,000 U/mL)/streptomycin (10,000 
µg/mL) solution, 0.25% trypsin–1 mM EDTA solution, Dulbecco’s phosphate-buffered saline, and geneticin (G418) were obtained from Life Technologies (Grand Island, NY). Fetal bovine serum was obtained from Hyclone (Logan, UT), and Matrigel was obtained from BD Biosciences (Bedford, MA). Δ4-Androstenedione, tamoxifen, hydroxypropyl cellulose, and Tween 20 were obtained from Sigma Chemical Company (St. Louis, MO). Letrozole (CGS 20267) was provided by Dr. D. Evans (Novartis Pharma, Basel, Switzerland). The pure antiestrogen faslodex (ICI 182,780) was supplied by Dr. A. Wakeling (AstraZeneca Pharmaceuticals, Macclesfield, U.K.).

Tumor Growth in Ovariectomized Female Athymic Nude Mice

All animal studies were performed according to the guidelines and approval of the Animal Care Committee of the University of Maryland School of Medicine. Female ovariectomized BALB/c athymic nude mice 4–6 weeks of age were obtained from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water ad libitum.

MCF-7Ca tumor xenografts were grown in the mice as previously described (10,11,16,17). MCF-7Ca cells were routinely maintained in Dulbecco’s modified Eagle medium with 5% fetal bovine serum, 1% penicillin/streptomycin solution, and 750 µg/mL G418. Subconfluent cells were scraped into Dulbecco’s phosphate-buffered saline, collected by centrifugation, and resuspended in Matrigel (10 mg/mL) at 2.5 × 10⁷ cells/mL. Each mouse received subcutaneous inoculations in two sites per flank with 100 µL of cell suspension. All mice were then injected daily with Δ4-androstenedione (100 µg/day) for the duration of the experiment. Tumor size was measured weekly with calipers, and tumor volume was calculated by the formula (4/3πr₁² × r₂), where r₁ is the smaller radius and r₂ is the larger radius. Treatments began when the tumors reached a measurable size (approximately 300 mm³), which was approximately 4 weeks after cell inoculation. Mice were then injected subcutaneously daily with the indicated drugs in addition to the Δ4-androstenedione supplement. Drugs were prepared as suspensions in 0.3% hydroxypropyl cellulose. Mice were treated for the indicated times, after which they were killed by decapitation and the trunk blood was collected. Tumors and uteri were excised, cleaned, weighed, and stored in liquid nitrogen for additional analysis.

First-Line Treatments With Letrozole and Tamoxifen In Vivo

Mice were divided into six groups (n = 20 per group) and received Δ4-androstenedione supplement (control, 100 µg/day) or Δ4-androstenedione supplement (100 µg/day) along with one of the following daily subcutaneous treatments: letrozole (10 µg/day); tamoxifen (100 µg/day); letrozole (10 µg/day) plus tamoxifen (100 µg/day); letrozole (10 µg/day) and tamoxifen (100 µg/day) on 4-week rotations, beginning with letrozole; and tamoxifen (100 µg/day) and letrozole (10 µg/day) on 4-week rotations, beginning with tamoxifen. The dose of letrozole used had been determined to be maximally effective in reducing tumor volume in several previous experiments (11). The dose of tamoxifen used was chosen because, in earlier studies (18,19), doses of tamoxifen of 60–500 µg/day showed equivalent tumor suppression. In addition, a dose of 100 µg/day led to optimal tumor growth suppression in previous mouse studies (18,19).

Second-Line Treatments With Letrozole and Tamoxifen In Vivo

After the tumors had doubled in volume and were unresponsive to the first-line therapy with tamoxifen, letrozole, or tamoxifen plus letrozole (at 16, 34, and 20 weeks, respectively; see “Results”), mice were divided into groups for second-line treatments, which were given for 12 weeks. Mice treated with tamoxifen as first-line therapy were divided into three groups for continued treatment with tamoxifen or for second-line treatment with letrozole or tamoxifen plus letrozole. Mice treated with tamoxifen plus letrozole as first-line therapy were divided into three groups for continued treatment with tamoxifen or letrozole or for second-line treatment with fulvestrant (1 mg/day) or tamoxifen. Tumors were monitored for up to week 46. As above, the dose of fulvestrant (1 mg/day) used in second-line therapy was the same as previously used and found to be optimally effective (19).

Measurement of Serum Letrozole and Tamoxifen Levels

Serum letrozole levels were measured as previously described (20). Briefly, letrozole was extracted from serum by mixing it with 100% diethyl ether. After vortexing and centrifugation at 10,000g for 10 minutes at 4°C, the top layer was removed, solvent was evaporated, and the extract was reconstituted in 1 mL of methanol. Levels of letrozole were measured by high-performance liquid chromatography using a Waters fluorescence detector (Waters, Milford, MA) (λ excitation = 230 nm; λ emission = 295 nm). Letrozole was separated at a flow rate of 1.5 mL/minute in a Waters Novapak 10-µm C18 column (3.9 mm × 15 cm) using a mobile phase of 0.01 M phosphate buffer:acetonitrile (70:30). The calibration curve was constructed using letrozole at concentrations ranging from 0.1 to 1000 ng/mL (r² = .99) in mouse plasma (Equitech-Bio, Kerrville, TX).

Serum tamoxifen levels were measured as previously described, with some modifications (21). Briefly, tamoxifen was extracted from serum by mixing it with 2% butanol in hexane (v/v). After vortexing and centrifugation at 10,000g for 10 minutes at 4°C, the top layer was removed, air-dried, and reconstituted in 1 mL of methanol. Prior to analysis by high-performance liquid chromatography using a mobile phase of methanol:water:triethylamine (93:7:0.01), 300 µL of the sample was exposed to short wavelength UV light (254 nm) at a distance of 10 cm for 1 minute in a box lined with aluminum foil. Tamoxifen was separated in a 5-µm ODS column (4.6 mm × 25 cm; Phenomenex Beckman, Torrance, CA) at a flow rate
of 1.0 mL/minute (λ excitation = 260 nm; λ emission = 375 nm). The calibration curve was constructed using tamoxifen at concentrations ranging from 5 to 1000 ng/mL (r² = .99) in mouse serum.

**Statistical Analysis**

Linear mixed-effects models were fitted to estimate average tumor weight and volume within each treatment group. Each experiment was analyzed separately. Weights of multiple tumors were obtained for each mouse after the mice were killed. The mean effect of treatment in each group and random effects for each mouse within a group were estimated. Data on tumor volume were longitudinal and unbalanced. The duration of treatment varied across the treatment groups. It was not possible to measure the same number of multiple tumors per mouse at all time points. In addition, diagnostic plots suggested that models of exponential growth were appropriate to the tumor growth data. Therefore, linear mixed-effects models were fitted to the natural logarithm of tumor volume over time. This approach allows an exponential parameter controlling the rate of growth to be estimated for each of these treatment groups, with the random effects being estimated for each subject in a group (22).

The general linear models technique was used to analyze the uterine weight data, which indicate endogenous estrogen levels. The treatment groups were compared at the .05 level of significance.

All hypothesis tests were two-sided. Adjustments for multiple comparisons were made by using either Tukey’s or Dunnett’s procedure (23,24). Results are presented as treatment mean and/or difference in the treatment means with the corresponding 95% confidence intervals (CIs).

**RESULTS**

**Growth Responses to First-Line Treatments With Letrozole and Tamoxifen In Vivo**

Mice were divided into six groups (n = 20 per group) and all received Δ4-androstenedione supplement. There was no statistically significant difference between tumor volumes of the groups at the start of treatment (week 0) (data not shown). The groups were administered either no drug treatment (control), treatment with letrozole (10 µg/day), treatment with tamoxifen (100 µg/day), treatment with letrozole plus tamoxifen at the same doses, treatment with letrozole alternating with tamoxifen on a 4-week rotation, and treatment with tamoxifen alternating with letrozole on a 4-week rotation. MCF-7Ca tumors in the control mice doubled in volume after 3–4 weeks (Table 1) and increased about sixfold in volume in 8 weeks (Fig. 1, A). The estimated mean tumor weight at week 8 was 843.7 mg (95% CI = 530.4 to 1157.0 mg) (Fig. 2, A). The mean wet uterine weight of the controls was 49.0 mg (95% CI = 40.5 to 57.5 mg), much higher than the average weight of the atrophic uterus (approximately 10 mg) of ovarietomized mice, indicating that aromatase in the tumors was functional and producing sufficient estrogen to stimulate uterine growth in the ovarietomized mice.

Tumor growth in tamoxifen-treated mice was relatively static compared with that in the control group for the first 8 weeks of treatment (Fig. 1, A and B). Thereafter, tumors started to proliferate slowly, doubling in volume after 16 weeks of treatment (Table 1). Tumor volumes were statistically significantly larger

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (in weeks) needed to increase the total tumor volume by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ4-Androstenedione (control)</td>
<td>3–4</td>
</tr>
<tr>
<td>Tamoxifen (100 µg/day)</td>
<td>16</td>
</tr>
<tr>
<td>Tamoxifen (100 µg/day) to letrozole (10 µg/day)</td>
<td>17–18</td>
</tr>
<tr>
<td>Tamoxifen (100 µg/day) plus letrozole (10 µg/day)</td>
<td>18</td>
</tr>
<tr>
<td>Letrozole (10 µg/day) to tamoxifen (100 µg/day)</td>
<td>22</td>
</tr>
<tr>
<td>Letrozole (10 µg/day)</td>
<td>34</td>
</tr>
</tbody>
</table>

*The control group received only Δ4-androstenedione and vehicle; all other groups received Δ4-androstenedione and the treatment drugs specified.

in the tamoxifen treatment group than in the letrozole treatment group at 28 weeks; the estimated difference in treatment effect (between tamoxifen and letrozole) after log-transformation was 0.8 (95% CI = 0.5 to 1.1) (P < .001).

Uterine weight was different across the six treatment groups (P < .001). At week 8, the mean uterine weight in the control group was 49.0 mg and that in the letrozole group was 13.5 mg (difference = 35.5 mg, 95% CI = 4.8 to 66.2 mg). The results indicate that letrozole indeed inhibited estrogen production.

Tamoxifen treatment was associated with increased uterine weight after week 4, and uterine weight in tamoxifen-treated mice remained high for the duration of the experiment (Fig. 2, A and B). After 28 weeks, mean uterine weight in the tamoxifen-treated mice was 60.0 mg (95% CI = 44.7 to 73.5 mg) (Fig. 2, A) and that in the control mice was 49.0 mg (95% CI = 45.1 to 52.9 mg). The average uterine weight in the group treated with letrozole plus tamoxifen was consistently higher than that in mice that received letrozole alone (Fig. 2, B). For instance, at week 12, the mean uterine weight in the letrozole plus tamoxifen group was 100.4 mg and that in the letrozole-alone group was 88.8 mg (difference = 81.6 mg, 95% CI = 56.7 to 106.5 mg). The finding of higher uterine weights in the letrozole plus tamoxifen group than in the letrozole-alone group suggests that the agonistic effect of tamoxifen on the uterus is evident when endogenous estrogen levels are reduced by letrozole treatment. This is consistent with the finding that serum levels of tamoxifen had stabilized by 4 weeks of treatment and remained stable for the duration of the experiment.

As previously reported (16,17), letrozole induced marked regression of MCF-7Ca tumors (Fig. 1, A and B). In these experiments, tumor volume was reduced by 54% over the first 4 weeks of treatment. Although the tumors subsequently began to proliferate slowly in the presence of letrozole, tumor volumes did not return to their pretreatment level until after 18 weeks of treatment. Furthermore, they did not double in volume until after 34 weeks of treatment (Table 1; Fig. 1, A). Letrozole treatment was continued for 56 weeks before the experiment was terminated (Fig. 2, A); at that point, tumor weights were 497.0 mg (95% CI = 206.3 to 787.7 mg) and uterine weights were 13.5 mg (95% CI = −8.1 to 35.1 mg) (Fig. 2, A). The reduced uterine weights compared with those of controls reflect the low level of estrogen being produced by the tumors in the presence...
of letrozole (25) and also indicate that letrozole was not an estrogen agonist. The low uterine weights associated with letrozole treatment were evident by 4 weeks of treatment (Fig. 2, B) and remained low for the duration of the therapy. The half-life of letrozole in mice is reported to be 24 hours (26). Serum levels of the drug (in ng/mL) changed little from week 4 (mean = 835.4, 95% CI = 468.1 to 1202.7 ng/mL) to week 56 (mean = 912.9, 95% CI = -1275.8 to 3101.6 ng/mL) (Table 2), indicating that letrozole levels were sufficient to maintain estrogen suppression throughout the experiment. Levels of tamoxifen were similar in serum from mice treated with letrozole and tamoxifen combined and from mice treated with tamoxifen alone (Table 3).

Tumors in mice treated with a combination of tamoxifen plus letrozole also regressed over the first 3 weeks of treatment (Fig. 1, A) but to a lesser extent than tumors treated with letrozole alone. After 3 weeks of treatment, tumors treated with the combination had regressed by 23%, but after 18 weeks of treatment, they had doubled in volume (Table 1; Fig. 1, A).

Fig. 1. Effects of letrozole (Let; 10 μg/day) and tamoxifen (Tam; 100 μg/day) and their combined or alternating treatment on the growth of MCF-7Ca breast tumor xenografts in female ovariectomized athymic nude mice. Mice were inoculated with MCF-7Ca human breast cancer cells at two sites per flank and were supplemented daily with Δ4-androstenedione (Δ4A; 100 μg/day) until mean tumor volumes were approximately 300 mm³. Mice were then divided into groups (n = 20 per group) and injected subcutaneously daily with letrozole (10 μg/day) and/or tamoxifen (100 μg/day) in addition to the Δ4-androstenedione supplement. Tumor volumes were measured weekly and are expressed as the percent change relative to the initial tumor volume. All groups were compared in one experiment. For simplicity, the results are shown in two panels. A) Long-term treatment with letrozole, tamoxifen, neither drug, or a combination of the two. Treatment with letrozole was statistically significantly better than the other treatments at 16 weeks. Tumor volumes were statistically significantly larger in the tamoxifen treatment group than in the letrozole treatment group at 28 weeks; the estimated difference in mean treatments (between tamoxifen and letrozole) after log-transformation was 0.8 (95% confidence interval = 0.5 to 1.1). B) Long-term treatment with alternating 4-week cycles of letrozole and tamoxifen, beginning with letrozole (Let to Tam) or with tamoxifen (Tam to Let), or with letrozole or tamoxifen alone. * indicates points at which the treatments were alternated from letrozole to tamoxifen and vice versa. At the end of the experiment (week 28), tumor volumes in mice treated with alternating letrozole and tamoxifen were statistically significantly larger than those in mice treated with letrozole alone (estimated mean difference in log-transformed tumor volume was 0.87 (95% confidence interval = 0.51 to 1.23).
Table 2. Serum levels of letrozole in MCF-7Ca xenograft mice treated with various combinations of tamoxifen and letrozole*

<table>
<thead>
<tr>
<th>Treatment (weeks)</th>
<th>Avg. letrozole, ng/mL serum (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ4-Androstenedione (8)</td>
<td>ND</td>
</tr>
<tr>
<td>Long-term letrozole (4)</td>
<td>835.4 (468.1 to 1202.7)</td>
</tr>
<tr>
<td>Long-term letrozole (8)</td>
<td>836.7 (760.4 to 913.0)</td>
</tr>
<tr>
<td>Long-term letrozole (12)</td>
<td>867.7 (337.7 to 1397.7)</td>
</tr>
<tr>
<td>Long-term letrozole (32)</td>
<td>868.0 (−922.8 to 2658.8)</td>
</tr>
<tr>
<td>Long-term letrozole (56)</td>
<td>912.9 (−1275.8 to 3101.6)</td>
</tr>
<tr>
<td>Long-term letrozole (56)</td>
<td>ND</td>
</tr>
<tr>
<td>Long-term letrozole (34) to tamoxifen (35–46)</td>
<td>ND</td>
</tr>
<tr>
<td>Long-term tamoxifen (28)</td>
<td>ND</td>
</tr>
<tr>
<td>Long-term tamoxifen (16) to letrozole (17–28)</td>
<td>818.1 (698.6 to 937.6)</td>
</tr>
<tr>
<td>Long-term tamoxifen (16) to tamoxifen plus letrozole (17–28)</td>
<td>638.4 (−607.2 to 1884.0)</td>
</tr>
<tr>
<td>Long-term tamoxifen plus letrozole (32)</td>
<td>643.2 (59.8 to 1226.6)</td>
</tr>
<tr>
<td>Long-term tamoxifen plus letrozole (20) to tamoxifen (21–32)</td>
<td>862.0 (168.0 to 1556.0)</td>
</tr>
<tr>
<td>Long-term tamoxifen plus letrozole (20) to tamoxifen (21–32)</td>
<td>ND</td>
</tr>
<tr>
<td>Letrozole to tamoxifen (12)†</td>
<td>851.6 (289.8 to 1413.4)</td>
</tr>
<tr>
<td>Letrozole to tamoxifen (20)†</td>
<td>871.2 (328.5 to 1413.9)</td>
</tr>
<tr>
<td>Tamoxifen to letrozole (28)†</td>
<td>889.2 (314.7 to 1463.7)</td>
</tr>
<tr>
<td>Tamoxifen (16) to letrozole (17–28)†</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Drugs were given at the following doses: letrozole, 10 μg/day; tamoxifen, 100 μg/day; fulvestrant, 1 mg/day. All mice received Δ4-androstenedione. Levels of letrozole were measured in serum obtained from animals 2 hours after final dosing. CI = confidence interval; ND = not detectable.
†All mice receiving alternating treatment starting with letrozole were killed after a course of letrozole.
‡All mice receiving alternating treatments starting with tamoxifen were killed after a course of tamoxifen.

Thus, letrozole used alone was statistically significantly more effective than tamoxifen used in combination with tamoxifen. After 16 weeks of treatment, the estimated difference in log-transformed tumor volume was 0.71 (95% CI = 0.42 to 0.99) (P < .001) (Fig. 1, A). By week 20, tumor volumes of mice treated with the combination of tamoxifen and letrozole continued to be greater than those of mice treated with tamoxifen alone, with an estimated difference in log-transformed tumor volumes of 0.80 (95% CI = 0.40 to 1.20) (P < .001) (Fig. 1, A). Tumors of mice treated with tamoxifen plus letrozole and/or with tamoxifen alone continued to increase in volume.

Uterine weights were compared in mice that were killed and autopsied at various times during treatment (Fig. 2, A and B). In mice treated with the combination of tamoxifen plus letrozole, uterine weights were similar to those in mice treated with tamoxifen. In mice treated with letrozole, uterine weights were smaller than those in all groups that received tamoxifen (for example, at week 12, P = .001). In mice treated with letrozole plus tamoxifen, estrogen production was inhibited by letrozole, yet uterine weights were similar to the uterine weights in controls that were receiving estrogen. This result clearly demonstrates the estrogenic effect of tamoxifen on the mouse uterus.

It has been reported (27,28) that treatment of patients with tamoxifen and letrozole combined results in a reduction of 37.6% in serum letrozole levels (P < .001) but does not affect serum tamoxifen levels. In our study, mice treated with letrozole alone for 32 weeks had a mean serum letrozole level of 868.0 ng/mL, whereas serum letrozole levels in mice treated with the combination was 643.2 ng/mL (difference = 224.8, 95% CI = −412.5 to 862.2) (Table 2). Serum levels were not different between these two treatment groups (P = .27). We had a sufficient amount of serum to measure levels of tamoxifen in only a small number of animals. These measurements, however, indicated a reduction of only 11% in the level of tamoxifen, from 427.6 ng/mL in mice treated with tamoxifen alone to 382.4 ng/mL in mice treated with tamoxifen plus letrozole (difference = 45.2 ng/mL, 95% CI = −150.6 to 241.0 ng/mL) (Table 3). The difference is not statistically significant (P = .43).

Tumors in mice that were treated with letrozole for 4 weeks and then treated with tamoxifen and letrozole alternately over 4-week intervals initially regressed during the first 4 weeks of letrozole treatment (Fig. 1, B). However, when the therapy was switched from letrozole to tamoxifen (weeks 5–8), the tumors proliferated rapidly for 2 weeks before growth stabilized. When the therapy was switched back to letrozole (weeks 9–12), the tumors regressed again, but for only 1 week, and then they proliferated slowly. Tamoxifen therapy throughout weeks 13–16 resulted in tumor proliferation. The pattern of response (i.e., initial regression followed by proliferation) was repeated for letrozole for weeks 17–24, but during weeks 24–28, tumors continued to proliferate with tamoxifen treatment. After four cycles of letrozole alternated with three cycles of tamoxifen, the tumors had acquired the ability to proliferate in the presence of both therapies. At the end of the experiment (i.e., at 28 weeks), tumor volumes in mice treated with alternating letrozole and tamoxifen were statistically significantly larger than those in mice treated with letrozole alone (difference in log-transformed tumor volume = 0.87, 95% CI = 0.51 to 1.23) (P < .001 at 16 weeks). Tumor weights were determined at autopsy. After 28 weeks, the mean tumor weight in the letrozole alternating with tamoxifen group was 280.2 mg and that in the tamoxifen alternating with letrozole group was 931.8 mg (difference = 651.6 mg, 95% CI = 143.0 to 1148.6 mg) (Fig. 2, A).

A similar pattern of tumor proliferation and regression was observed in mice that were treated first with tamoxifen for 4 weeks and then treated alternately with letrozole and tamoxifen over 4-week intervals (Fig. 1, B). Tumor growth was relatively static during the first 4 weeks of tamoxifen therapy. When switched to letrozole (weeks 5–8), tumors initially regressed for 2 weeks and then proliferated slowly. Tamoxifen therapy (during weeks 9–12) was associated with rapid proliferation. When switched back to letrozole (weeks 13–16), the tumors again regressed for 2 weeks before proliferating thereafter on either drug. Ultimately, tumors were able to proliferate in the presence of letrozole after three cycles of tamoxifen. However, when the first treatment in the rotation was tamoxifen, tumors doubled in volume in 22 weeks compared with 17–18 weeks when the first course of treatment was letrozole. At 28 weeks, tumor volumes

Table 3. Serum levels of tamoxifen in MCF-7Ca mice treated with tamoxifen and letrozole*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Avg. tamoxifen, ng/mL serum (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamoxifen</td>
<td>427.6 (76.8 to 778.4)</td>
</tr>
<tr>
<td>Tamoxifen plus letrozole</td>
<td>382.4 (−65.0 to 829.8)</td>
</tr>
<tr>
<td>Δ4-Androstenedione</td>
<td>ND</td>
</tr>
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</table>

*All mice received Δ4-androstenedione. After 28 weeks, levels of tamoxifen were measured in serum obtained from two mice per group 2 hours after the final dosing. Samples were assayed in duplicate, and assays were repeated twice. CI = confidence interval; ND = not detectable.
in mice treated with tamoxifen alternating with letrozole were statistically significantly larger than those in mice treated with letrozole (difference in log-transformed tumor volume = 1.29, 95% CI = 0.82 to 1.76) (P<.001). However, treatment with letrozole alone was more efficient at reducing tumor volume than treatment with tamoxifen alone, with a mean difference in log-transformed tumor volume of 0.50 (95% CI = 0.01 to 0.90) (Fig. 1, B). Nevertheless, compared with therapy with letrozole alone, neither of the alternating regimens prolonged time to tumor progression (i.e., the time when tumors begin to grow on treatment).

**Effect of Second-Line Therapies on Tumor Growth**

*In Vivo*

After 16 weeks, tumors in the tamoxifen-treated mice had doubled in volume and were deemed unresponsive to therapy. Because some mice had been autopsied at earlier time points, 14 mice remained in the tamoxifen group. These mice were divided into three groups (four mice, five mice, and five mice) with similar (and not statistically significantly different) total tumor volumes. The first group was treated for another 12 weeks with tamoxifen, and the other groups were administered second-line treatment with letrozole or a combination of tamoxifen plus letrozole for an additional 12 weeks (Fig. 3, A). The tamoxifen-treated tumors continued to proliferate for the duration of the experiment, and tumor volumes in that group increased 3.6-fold over the next 12 weeks. Tumor growth in the mice switched to letrozole decreased during the first week, stabilized for the next 2 weeks, and then increased slowly. Over the last 3 weeks of letrozole treatment, tumors grew more rapidly and volumes increased by 1.6-fold. Therefore, letrozole was not as effective for second-line therapy as it was for first-line treatment (mean difference in log-transformed tumor volume at 28 weeks = 1.13, 95% CI = 0.66 to 1.60) (P<.001).

At the end of the experiment, i.e., at 28 weeks (Fig. 3, A), the average tumor volume in mice treated with tamoxifen was greater than that in mice treated with tamoxifen plus letrozole as second-line therapy, but the difference was not statistically significant. However, the differences in mean tumor weight and tumor volume were statistically significantly different between mice receiving second-line letrozole and mice continuing on tamoxifen. The difference in mean tumor weight was 1061.8 mg (95% CI = 84.1 to 2207.7 mg, P<.001). Serum letrozole levels in the second-line treatment group were similar to those in mice that received letrozole as first-line treatment (Table 2), suggesting that estrogen production was equally inhibited with first- and second-line letrozole treatment. Reduced mean uterine weights in the group treated with letrozole (22.4 mg) compared with those in the group treated with tamoxifen (60 mg) (difference = 37.6 mg, 95% CI = 26.5 to 48.7 mg) indicate that estrogen production was effectively inhibited by letrozole. Tumors in the mice switched from tamoxifen to the second-line combination of tamoxifen plus letrozole therapy also regressed during the first week of treatment, but regression was less pronounced compared with that in mice receiving second-line letrozole alone. Thereafter, tumors proliferated slowly, increasing in volume by 2.5-fold over the next 11 weeks of treatment.

The tumors of mice that received first-line treatment with the combination of tamoxifen plus letrozole doubled in volume after 18 weeks. At week 20 of treatment, the mice remaining in this group (n = 9) were divided into three groups (of three mice each) with similar (and not statistically significantly different) tumor volumes. One group was continued on tamoxifen plus letrozole for another 12 weeks, and the other groups received second-line treatment with either tamoxifen or letrozole for 12 weeks (Fig. 4, A). Tumors of mice treated with the combination continued to proliferate for the duration of the experiment, with volumes increasing by an additional 3.3-fold over the 12 weeks of continued combination treatment. Tumor volumes in the mice switched to second-line tamoxifen increased 2.8-fold over the same 12 weeks. Tumor and uterine weights in the mice switched to tamoxifen were similar to those in the mice that remained on the combination treatment (Fig. 4, B). In contrast, tumors in
mice switched to letrozole alone regressed for the first 2 weeks before doubling in volume during the following 12 weeks of treatment. Moreover, mean weights of tumors in mice switched to letrozole were lower than those in mice treated with tamoxifen or with the combination of tamoxifen plus letrozole (difference between letrozole-treated mice and mice receiving combination treatment = 362.2 mg, 95% CI = 17.4 to 706.6 mg) (Fig. 4, B). Letrozole used as first-line treatment was statistically significantly better at suppressing tumor volumes than when used sequentially or in combination (P < .001 and P = .02, respectively) at week 32 (Fig. 4, A). Uterine weights were also lower with letrozole treatment than with combination treatment (difference = 45.7 mg, 95% CI = 12.5 to 78.9 mg) (P = .006), indicating that letrozole was inhibiting intratumoral estrogen production, even though tumors progressed on the treatments. After 34 weeks of first-line letrozole therapy, the 12 remaining mice were divided into three groups with similar (i.e., not statistically significantly different) tumor volumes for continued treatment with letrozole (n = 3), second-line treatment with tamoxifen (n = 5), or second-line treatment with the pure antiestrogen fulvestrant (n = 3) for the next 12 weeks (Fig. 5). (The combination of tamoxifen plus letrozole was not examined because it had become clear that this combination was providing results similar to treatment with tamoxifen alone.) Tumor volumes in mice switched to second-line tamoxifen or fulvestrant therapy continued to proliferate, with no reduction in tumor growth rate relative to that in mice continued on letrozole treatment. No statistically significant differences in tumor weights among the three treatment groups were found at the end of the experiment. Thus, antiestrogens may not be effective second-line treatment for tumors progressing on letrozole therapy. However, as expected, fulvestrant was less estrogenic than tamoxifen because uteri were not hyperplastic and weighed statistically significantly less than uteri in mice receiving second-line tamoxifen treatment (mean difference = 65.9 mg, 95% CI = 40.7 to 91.0 mg).

**DISCUSSION**

Our data indicate that first-line treatment of an antiestrogen- and aromatase inhibitor–responsive breast cancer model with the aromatase inhibitor letrozole may lead to a better therapeutic outcome than treatment with tamoxifen by extending time to tumor progression. For example, letrozole prolonged the time to
tumor progression approximately twofold compared with tamoxifen (34 weeks versus 16 weeks). We also found that combining tamoxifen with letrozole or alternating 4-week courses of tamoxifen and letrozole did not extend time to tumor progression. Indeed, compared with tamoxifen therapy alone or tamoxifen alternating with letrozole or letrozole alternating with tamoxifen, letrozole therapy alone led to a statistically significant inhibition of tumor growth \((P<.001)\) and delayed the time to tumor progression.

We also found that the combination of tamoxifen plus letrozole was equivalent to tamoxifen alone. This result parallels the recently published findings of the Anastrozole, Tamoxifen, Alone or in Combination (ATAC) trial (6). The ATAC trial compared the nonsteroidal aromatase inhibitor anastrozole alone or in combination with tamoxifen with tamoxifen alone as adjuvant therapies for postmenopausal patients with early-stage breast cancer. After a median follow-up of 33.3 months, disease-free survival was 89.4% in the patients receiving anastrozole and was statistically significantly higher than the disease-free survival \((87.4\%)\) in patients receiving tamoxifen (odds ratio \([OR] = 0.83, 95\% CI = 0.71 to 0.96; P = .013\)). Disease-free survival after combination treatment \((87.2\%)\) was not statistically significantly different from that after treatment with tamoxifen alone \((OR = 1.02, 95\% CI = 0.89 to 1.18; P = .8)\). Moreover, the incidence of contralateral breast cancer was statistically significantly lower in patients receiving anastrozole than in patients receiving tamoxifen or the combination \((OR = 0.42, 95\% CI = 0.22 to 0.79; P = .007)\). These results are consistent with those of our previous studies of both anastrozole and letrozole in combination with tamoxifen (17), as well as with the results of this study.

Two possibilities could explain why combining a nonsteroidal aromatase inhibitor with tamoxifen did not provide advantages over treatment with the aromatase inhibitor alone. One possibility is that the combination of tamoxifen plus an aromatase inhibitor results in reduced serum letrozole levels in mice. Indeed, statistically significant reductions in serum letrozole levels of approximately 37\% \((P<.001)\) (27,28) and in serum anastrozole levels of 27\% \((29)\) have been observed in patients receiving these drugs in combination with tamoxifen. However, the reductions in plasma levels of these aromatase inhibitors in patients receiving combination therapy did not result in changes in circulating estrogen levels, which remained low (27). In our study, when letrozole and tamoxifen were combined, the serum levels of both declined slightly but not statistically significantly, by 27\% for letrozole and by 11\% for tamoxifen. It is clear that, in mice receiving combination therapy, the available levels of letrozole were sufficient to inhibit estrogen production, as shown by a reduction in uterine weight. Moreover, in previous studies (16,17), we found that letrozole administered to mice at half the dose used in this study \((i.e., 5\ \mu g/day)\) had statistically significant antitumor activity. It should also be noted that tamoxifen at lower doses \((e.g., 60\ \mu g/day)\) has been found to have considerable antitumor activity in mice (16).

A second possible explanation for the lack of improvement with the combination is that the elimination of 99\% of estrogens from the body with letrozole treatment \((30)\) causes the partial estrogen agonistic effects of tamoxifen to become apparent \((31,32)\). Even though the estrogen level in the xenograft tumors may be higher than the concentration in patients’ tumors, the dose of tamoxifen used \((100\ \mu g/day)\) was clearly effective and led to a response equivalent to that seen with 500 \(\mu g/day\) of tamoxifen. Thus, when levels of estrogen were suppressed by letrozole in the combination group \((Fig. 1, A)\), tumor volumes were similar to those in the group treated with tamoxifen alone but not to those in the group treated with letrozole alone. The agonist effects of tamoxifen were also apparent from the finding of hyperplastic uteri in mice treated with the combination of tamoxifen and letrozole compared with decreased uterine weights in mice treated with letrozole alone \((Fig. 2, B)\). In addition, tumor volumes increased in the alternating treatment regimens when tamoxifen was administered after 4 weeks of letrozole therapy, regardless of whether treatments began with letrozole or with tamoxifen. This result contrasts with the effects of tamoxifen administered as a first-line treatment, in which tumor growth was held static for the first 8 weeks of treatment, suggesting that the agonist effect of tamoxifen is apparent only when estrogen is absent. The failure of both alternating regimens to delay time to tumor progression relative to letrozole alone appears to be due to the fact that when therapy was alternated from letrozole to tamoxifen, tumor growth increased rapidly. It remains to be determined whether these findings will be confirmed when the results of the Breast International Group/Femera-Tamoxifen (BIG/FEMTA) trial are released. This adjuvant trial is comparing the effects of alternating letrozole for 2–3 years with tamoxifen for 2–3 years and alternating tamoxifen for 2–3 years with letrozole for 2–3 years \([reviewed in (7)]\). The preclinical data presented here raise the possibility that, following 2–3 years of letrozole therapy, tumors of some patients may recognize tamoxifen as an estrogen agonist, causing the treatment to be inferior to single-agent therapy with letrozole.

In addition to determining the efficacy of combination therapy, our second aim was to determine the optimal sequence of endocrine treatments and, in addition, the effect of second-line therapies on tumor progression. When tumors doubled in volume after 16 weeks of tamoxifen treatment, some mice were assigned to treatment with letrozole or a combination of tamoxifen plus letrozole. Second-line therapy with letrozole alone proved better than tamoxifen in that with the combination, possibly because tumors recognized tamoxifen as a weak estrogen that stimulates tumor growth to some extent. It is interesting to note that second-line treatment with letrozole was not as effective as first-line treatment with letrozole. First-line letrozole treatment caused tumor regression for the first 4 weeks of treatment, after which tumors grew at a very slow rate. By contrast, second-line letrozole treatment after tamoxifen caused regression for only 1 week before the tumors began to proliferate at a faster rate. A similar finding has been observed in patients \((4,33,34)\). When letrozole was compared with megestrol acetate and aminoglutethimide as second-line therapy for tamoxifen-refractory breast cancer, the objective response rates were 24\% and 19\%, respectively \((33,34)\). However, when letrozole was compared with tamoxifen as first-line treatment for advanced breast cancer, the overall response rate was 32\% with letrozole versus 21\% with tamoxifen \((35)\). Anastrozole, when administered as first-line treatment, provides an objective response in 21\%–33\% of patients \((1–3)\), but when this drug was administered as second-line treatment for tamoxifen-refractory breast cancer, objective responses occurred in only 10\% of the patients \((36)\). Similar findings have been reported for exemestane \((5,37)\). The biologic mechanisms underlying these results are unknown, but the data
suggest that tumors progressing on tamoxifen therapy have acquired some degree of estrogen-independent growth.

Second-line letrozole therapy also provided a better response rate than tamoxifen alone. Recent data from the M17 trial (38) suggest that patients who received letrozole following 5 years of tamoxifen treatment for early-stage breast cancer experienced fewer recurrences (75 among 2593 patients) than patients receiving placebo (132 among 2594 control subjects). This is an important advantage because there is no evidence that extending tamoxifen treatment beyond 5 years increases its benefits (8). Similarly, tumors progressing on the tamoxifen plus letrozole combination responded to second-line letrozole. Nevertheless, letrozole was statistically significantly better than tamoxifen at slowing tumor growth because, when switched to tamoxifen, tumors that received first-line treatment with the tamoxifen plus letrozole combination continued to proliferate rapidly. The clinical relevance of this finding may be that patients in the ATAC trial who progress while on the combination of anastrozole and tamoxifen might respond better to second-line therapy with anastrozole alone than to tamoxifen.

Tumors progressing on letrozole treatment at 34 weeks were switched to either tamoxifen or the pure antiestrogen faslodex. Surprisingly, neither tamoxifen nor faslodex was an effective second-line treatment for tumors actively progressing on letrozole. This finding is in contrast to our previous report, which indicated that both drugs, but especially faslodex, were effective second-line treatments for tumors that had progressed on letrozole (25). However, the experimental design of the two studies was different. In our previous study, tumors progressing on letrozole therapy were transplanted into new mice, and letrozole, but not the A4-androstenedione supplement, was withdrawn for 8 weeks until tumors were established. That study was designed to determine whether tumors that recur following limited aromatase-inhibitor therapy remain sensitive to second-line therapies with antiestrogens, and it found that both tamoxifen and fulvestrant treatment were effective at inhibiting tumor growth. That experiment was similar in some respects to the ATAC and BIG/FEMTA adjuvant trials, in which administration of the aromatase inhibitor is limited to 5 years. This study, by contrast, models advanced (metastatic) disease, in which patients relapsing on an aromatase inhibitor or an antiestrogen are switched directly to the other class of drug. The data from this study indicate that when advanced tumors were switched from an aromatase inhibitor directly to an antiestrogen, tumors continued to progress, with no statistically significant difference between any of the second-line treatment groups.

We (25) and others (39) have reported that long-term estrogen deprivation of MCF-7 cells in vitro is associated with increased expression of the estrogen receptor. Masamura et al. (40) reported that the high levels of estrogen receptor hypersensitize the cells to low levels of estrogens and consequently to the growth-inhibitory effects of pure antiestrogens. The data presented in our in vivo study (25) suggest that long-term estrogen deprivation with the aromatase inhibitor letrozole is not associated with increased levels of expression of estrogen receptors and that the MCF-7Ca tumor cells are not hypersensitive to estrogens or antiestrogens. In fact, we found that tumors receiving long-term letrozole treatment were growth refractory to second-line treatments with both tamoxifen and faslodex. Clinically, there is minimal response to tamoxifen as second-line therapy in advanced breast cancer patients refractory to letrozole, and survival with second-line letrozole is substantially longer than with second-line tamoxifen (35).

In summary, we have used a hormone-responsive xenograft model and shown that the aromatase inhibitor letrozole is superior, in terms of time to tumor progression, as a first-line treatment to the antiestrogen tamoxifen, to a combination of tamoxifen plus letrozole, and to therapies that alternate tamoxifen with letrozole (and vice versa). Letrozole was also determined to be an effective second-line treatment for tumors progressing on tamoxifen alone and on the combination. However, the antiestrogens tamoxifen and fulvestrant did not appear to be effective for tumors progressing on letrozole. Therefore, further studies are needed to determine the optimal second-line therapy for patients whose tumors progress after letrozole therapy.

**REFERENCES**


NOTES

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