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The intratumoral aromatase model: studies with aromatase inhibitors and antiestrogens☆

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Abstract

Aromatase inhibitors have now been approved as first-line treatment options for hormone-dependent advanced breast cancer. When compared to tamoxifen, these aromatase inhibitors provide significant survival and tolerability advantages. However, the optimal use of an aromatase inhibitor and tamoxifen remains to be established. To date, the intratumoral aromatase xenograft model has proved accurate in predicting the outcome of clinical trials. Utilizing this model, we performed long-term studies with tamoxifen and letrozole to determine time to disease progression with each of the treatment regimens. Aromatase-transfected MCF-7Ca human breast cancer cells were grown as tumor xenografts in female ovariectomized athymic nude mice in which androstenedione was converted to estrogen and stimulated tumor growth. When tumor volumes were approximately 300 mm^3, the animals were grouped for continued supplementation with androstenedione only (control) or for treatment with letrozole 10 μg per day (long-term), tamoxifen 100 μg per day (long-term), letrozole alternating to tamoxifen (4-week rotation), tamoxifen alternating to letrozole (4-week rotation), or a combination of the two drugs. Tumors of control mice had doubled in volume in 3–4 weeks. In mice treated with tamoxifen and the combination, tumor doubling time was significantly shorter (16 and 18 weeks, respectively) than with letrozole (34 weeks). Furthermore, alternating letrozole and tamoxifen treatment every 4 weeks was less effective than letrozole alone. Tumors doubled in 17–18 weeks when the starting treatment was tamoxifen and in 22 weeks when the starting treatment was letrozole. Tumors progressing on tamoxifen remained sensitive to second-line therapy with letrozole (10 μg per day). However, when mice with letrozole-resistant tumors were switched to antiestrogen treatment, tumors did not respond to tamoxifen (100 μg per day) or faslodex (1 mg per day). This suggests that advanced breast cancers treated with letrozole may be insensitive to subsequent second-line hormonal agents. Thus, although letrozole was determined to be an effective second-line treatment option for tumors progressing on tamoxifen, antiestrogen therapy does not appear to be effective for tumors progressing on letrozole. However, response to second-line treatment was observed in a model where tumors that had progressed on letrozole were transplanted to new mice. These tumors had been allowed to grow in the presence of supplemented androstenedione but absence of letrozole. This suggests that resistance to letrozole may be reversible, allowing tumors to respond to subsequent antiestrogens and letrozole.

Keywords: Aromatase; Antiestrogen; Tamoxifen

1. Introduction

Progression of hormone-sensitive breast cancer is a result of stimulation by estrogen acting through the estrogen receptor (ER). A larger proportion of postmenopausal breast cancer patients are women with estrogen receptor positive (ER+) tumors, even though there is a decline in their ovarian estrogen production. Nevertheless, estrogen synthesis via aromatization of mainly adrenal androgens continues in extragonadal sites, such as adipose tissue [1]. Although circulating levels are low in postmenopausal women, the breast itself produces estrogen levels that are four to six times higher than in the serum and that are equivalent to breast tissue levels in premenopausal women [2]. Thus, the local estrogen level in the tumor could be an important contribution influencing the proliferation of the tumor in hormone-sensitive breast cancer. Therefore, systemic treatments that inhibit the growth stimulus of estrogen are effective in providing long-term benefit to these women [3–5]. Over the last two decades, the use of the antiestrogen tamoxifen which blocks the action of estrogen at the estrogen receptor has proved to be a significant improvement in breast cancer treatment [3]. Reducing estrogen production with aromatase (estrogen synthetase) inhibitors has also been shown to be effective in breast cancer [6,7].

As several potent aromatase inhibitors have now become available for treatment, the relative activities of these

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compounds in controlling breast tumor growth in comparison to tamoxifen are of considerable interest. Some limitations of this antiestrogen are that while it is effective in controlling tumor progression, tamoxifen functions mainly as an estrogen agonist in other tissues, increasing the risk of strokes and occasionally leading to secondary tumors of the endometrium [3]. Selective aromatase inhibitors could be safer and more effective than antiestrogens since they do not have these agonist features. Aromatase inhibitors have already proved useful in treating patients with advanced breast cancer who have relapsed while on tamoxifen treatment [6,7]. Because of their different chemical structures and acting by a different mechanism from tamoxifen, aromatase inhibitors can result in secondary beneficial responses.

To study the effectiveness of using aromatase inhibitors and antiestrogens in different treatment strategies, we developed a xenograft model in the mouse that simulates to some extent the postmenopausal breast cancer patient with ER+ tumors [8,9]. In order to eliminate estrogen production under gonadotropin regulation, ovariectomized mice are utilized. However, as the mouse has no significant production of peripherally formed estrogen and low adrenal androgen production, MCF-7 human breast cancer cells stably transfected with the human aromatase gene (*MCF-7Ca*) [10] were utilized in the model. These cells convert supplemented androstenedione to estrogen to stimulate tumor growth. This model allows us to evaluate the effects of both antiestrogens and aromatase inhibitors on tumor growth and has provided valuable data that predicted the efficacy of aromatase inhibitors in breast cancer patients [11,12].

Three aromatase inhibitors have been approved for breast cancer treatment in the US. The triazole compound letrozole has been found to be superior to tamoxifen [13]. Anastrozole, a similar triazole compound, was at least as and possibly more effective than tamoxifen in ER+ patients [14,15] and had fewer side effects. These results are consistent with those from the tumor model [12]. Studies so far with the steroidal aromatase inhibitor, exemestane, suggest that this compound also may be more effective than tamoxifen [16]. However, the best strategy for the use of these compounds in the treatment of breast cancer remains to be determined and raise a number of intriguing questions. For example, would better efficacy be achieved by combining agents that inhibit estrogen synthesis with compounds that inhibit its actions? Also, could the development of overall resistance to these hormonal therapies be delayed by alternating their use? We have used the model to address these questions and to provide guidance in the design of future clinical trials.

2. Methods

2.1. Intratumoral aromatase model

MCF-7 cells transfected with the human aromatase gene (*MCF-7Ca*) were grown in MEM as described elsewhere [8,9]. When the culture was subconfluent, the 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) to make a cell suspension of 3×10^7 cells/ml. Ovariectomized female BALB/c mice (aged 4–6 weeks) were inoculated subcutaneously (s.c.) in four sites each with 0.1 ml of the cell suspension. Androstenedione, the substrate for aromatase, was supplemented throughout the experiment at a dose of 0.1 mg per mouse per day s.c. Tumor growth was measured with calipers weekly and tumor volumes were calculated according to the formula $(4/3)\pi r_1^2 r_2$ ($r_1 < r_2$). The animals were housed in a pathogen-free environment under controlled conditions of light, temperature, and humidity, and received food and water ad libitum.

When all tumors had reached a measurable size ($\sim$500 mm^3), usually 28–35 days after inoculation, animals were assigned to groups of four or five mice. Mice were then injected s.c. daily with the following aromatase inhibitors or antiestrogens, or a combination of these agents. Letrozole (CGS 20,267) (kindly provided by Dr. Dean Evans, Novartis, Basel, Switzerland), anastrozole (ZD 1033) (kindly provided by Dr. Michael Dukes, Zeneca Pharmaceuticals, Macclesfield, UK), and the antiestrogen tamoxifen (purchased from Sigma) were prepared for injection in 0.3% hydroxypropyl cellulose (HPC). Control animals received the vehicle 0.1 ml per mouse per day s.c. The ER down-regulator, faslodex (ICI 182,780) (kindly provided by Dr. A. Wakeling, Zeneca Pharmaceuticals) was injected in an oil preparation. Groups of mice injected with a combination of aromatase inhibitor and antiestrogen were administered the same doses as for each agent used alone. Tumor volumes were measured weekly. Animals were autopsied 4–6 h after the last injection. Tumors and uteri were removed from the mice, cleaned, weighed, and stored at −80 °C until analyzed. Tumors were analyzed by Western blots for ER, progesterone receptors (PR), and erbB2 using a monoclonal antibody for ER (Santa Cruz Biotechnology, CA), a rabbit polyclonal antibody for PR (Santa Cruz Biotechnology) and a rabbit polyclonal antibody for erbB2 (Upstate Biotechnology, NY).

3. Results

In this model, letrozole administered over a dose range of 0.5–10 μg per day for 9 weeks, causes a dose-dependent tumor response [17]. Maximal tumor inhibition occurred with 10 μg per day of letrozole [17,18]. Doses of the steroidal inhibitor, exemestane (50–250 μg per day) showed maximum growth suppression at 250 μg per day. Tamoxifen showed similar growth suppression at 500 μg per day. However, tamoxifen treatment increased uterine weight to a similar extent as occurred in the control animals [18]. Endogenous estrogen levels in the control mice were sufficient to maintain the uterus comparable to that of the intact mouse in diestrus.

In contrast, uterine weight was reduced by aromatase inhibitor treatment in comparison with those of controls and was not dose dependent.

The effect of anastrozole, letrozole or the estrogen receptor down-regulator fulvestrant on tumor growth was evaluated in a comparison experiment. Treatment with letrozole 10 μg per day was significantly better than anastrozole 10 or 60 μg per day and fulvestrant 5 mg per week. By 28 days of treatment, tumors treated with letrozole had regressed by nearly 80% from their initial size [11]. Anastrozole prevented tumor progression at 10 μg per day and reduced tumor volume <20% at 60 μg per day. Fulvestrant reduced tumor volume by nearly 30% of their initial size by day 28 [20]. These studies showed that in the tumor model letrozole was more effective than either anastrozole or fulvestrant. Current data in breast cancer patients also suggests that letrozole may be the most beneficial in first-line treatment [13].

3.1. Combining antiestrogens and aromatase inhibitors

Combining aromatase inhibitor and antiestrogen treatment to block estrogen synthesis and action is an appealing hypothesis. In order to determine whether these agents would act synergistically or additively, two 5-week studies were carried out with doses approximating the ED_{50}. However, the combination of anastrozole or letrozole with tamoxifen or fulvestrant was no more effective than the non-steroidal aromatase inhibitors alone [12]. A similar study was recently carried out with exemestane. The combination of exemestane and tamoxifen proved to be significantly better than either tamoxifen alone or the same dose of exemestane alone (Fig. 1) resulting in complete tumor growth suppression lasting for at least 8 weeks, whereas tamoxifen-treated tumors had more than doubled in this time.

In a recent study in the tumor model, treatment regimens of tamoxifen and letrozole were continued until tumors doubled in size. This was similar to assessing time to treatment failure in breast cancer patients. Animals were treated with doses of the single agent likely to cause greater tumor suppression. Letrozole at 10 μg per day and tamoxifen at 100 μg per day were administered alone and in the combination of the two agents. The duration of effective treatment was measured as a function of tumor volume expressed as the time to tumor doubling. This 35-week study confirmed that the combination of the non-steroidal aromatase inhibitor, letrozole, with tamoxifen was similar to tamoxifen alone but not as effective as treatment with the aromatase inhibitor alone (Fig. 2) [19,20]. Thus, tumors treated with tamoxifen

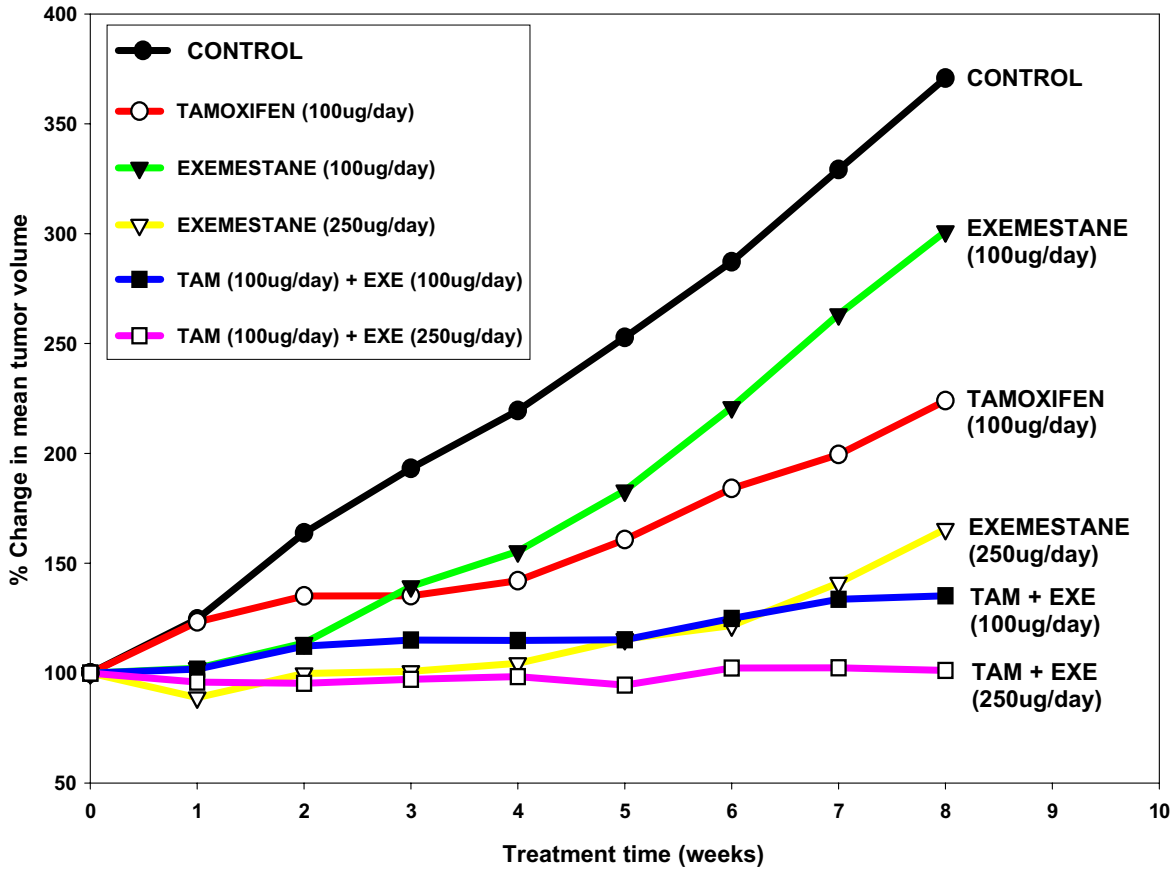

Fig. 1. The effect of exemestane and tamoxifen treatment on MCF-7Ca tumor doubling time.

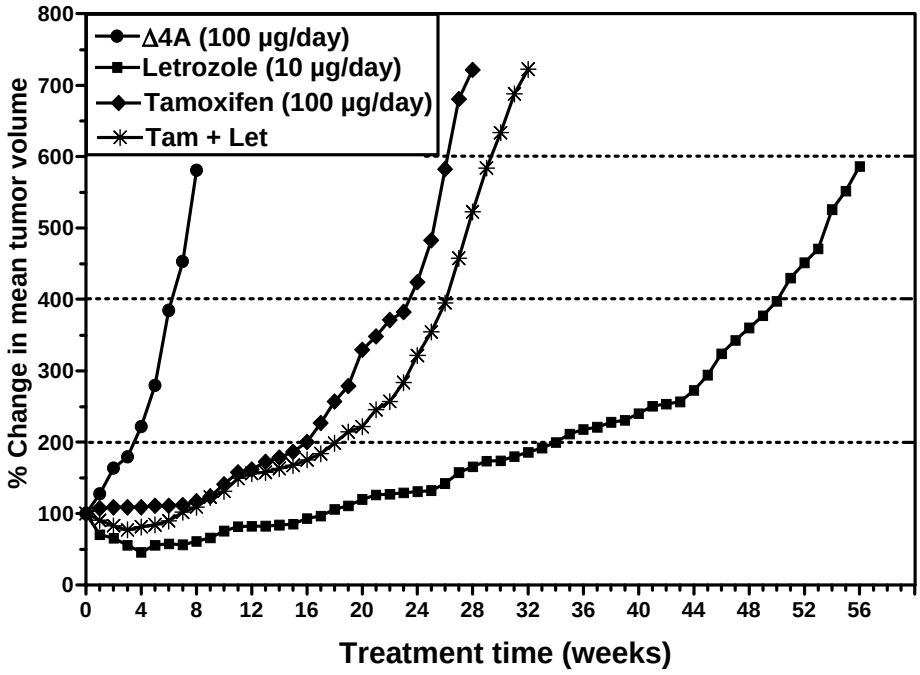

Fig. 2. The effect of combining tamoxifen and letrozole treatment on MCF-7Ca tumor doubling time.

alone had doubled in volume in 16 weeks, whereas tumors treated with tamoxifen plus letrozole in combination had doubled in volume in 18 weeks. However, tumor growth inhibition was much longer in letrozole-treated mice and remained suppressed for 35 weeks of treatment. Letrozole caused marked inhibition of tumor growth and also tumor regression. In comparison, tamoxifen treatment reduced tumor growth but did not cause regression in the mouse model. Thus, the non-steroidal aromatase inhibitor was more effective than in combination with tamoxifen or tamoxifen treatment alone. These results were confirmed in the recently reported ATAC (arimidex or tamoxifen alone or in combination) trial [21,22]. In this largest adjuvant trial carried out to date, anastrozole alone was a significantly better treatment than either tamoxifen or the combination of these agents.

3.2. Sequential treatment

Tumors had doubled in volume with tamoxifen treatment by 16 weeks [19,20]. The mice were then administered letrozole or letrozole plus tamoxifen. Tumor growth of mice treated with letrozole plus tamoxifen or letrozole alone was reduced after 2 and 4 weeks, respectively, compared to growth of those continued on tamoxifen. However, a better overall response was seen when the mice were treated initially with letrozole. This resulted in significantly better inhibition of tumor growth than all other treatments (Fig. 3). A similar finding has now been confirmed in the recent randomized trial in which patients had the choice of crossing over from tamoxifen to letrozole or vice versa or remaining on their initial treatment. Those who did not cross over, but remained on letrozole had the best outcome [20].

When tumors of letrozole-treated mice had doubled in volume after 35 weeks, the animals were divided into three groups and then administered either tamoxifen or faslodex or continued on letrozole. However, no tumor suppression occurred. Tumors grew more rapidly with tamoxifen and faslodex than those continued on letrozole treatment [19].

Analysis by Western blotting of tumors removed from the above treated mice at 4 and 8 weeks and subsequent times after treatment with tamoxifen and letrozole showed an initial increase in both estrogen and progesterone receptor levels at 4 weeks followed by a decline in receptor levels at later weeks (12–32 weeks). In contrast, erbB2 was markedly induced at 4 weeks and all subsequent weeks in both the tamoxifen- and letrozole-treated tumors. Thus, erbB2 was overexpressed in both responding and non-responding tumors [19].

3.3. Alternating aromatase inhibitor and antiestrogen treatment

In efforts to delay the development of tumor resistance and extend the duration of effective therapy, mice were alternated between tamoxifen and letrozole every 4 weeks (Fig. 4). Mice with measurable tumors were started on treatment with either tamoxifen (100 μg per day) or letrozole (10 μg per day) and then crossed over every 4 weeks to the other treatment [19,20]. Tumors doubled in tamoxifen-treated animals

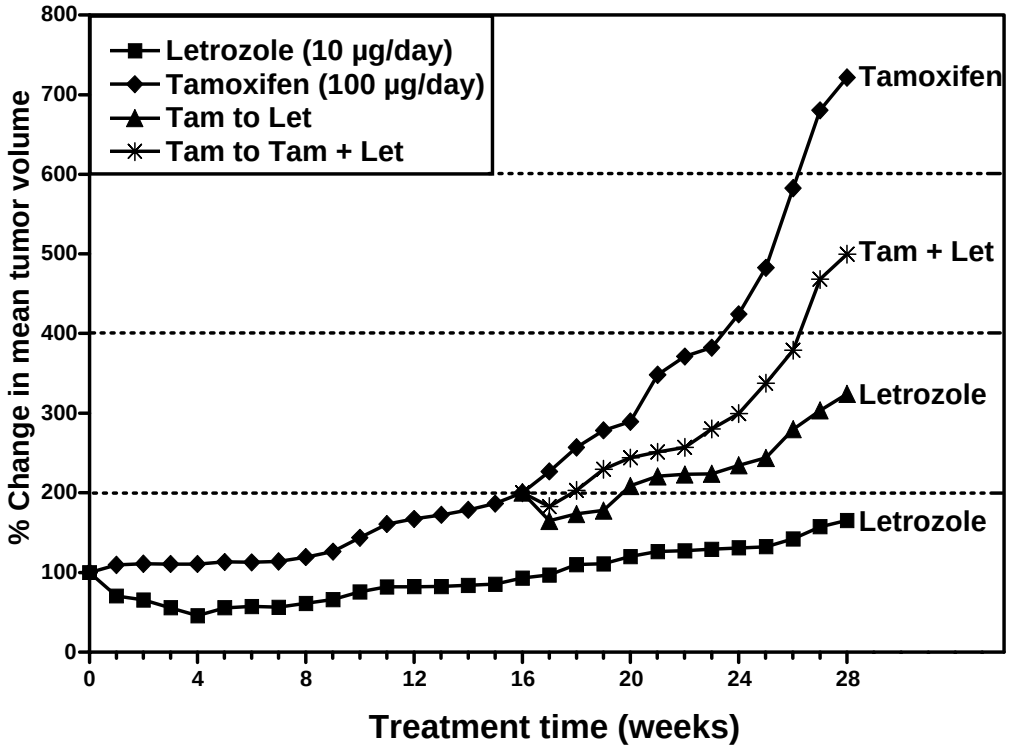

Fig. 3. The effect of sequential letrozole treatment on tamoxifen-treated MCF-7Ca tumor doubling time.

in 16 weeks. Tumor doubling time in animals started on tamoxifen treatment and crossing over to letrozole was delayed by only 2 weeks. Interestingly, starting animals on letrozole and alternating to tamoxifen delayed tumor doubling time to 22 weeks. However, tumor growth inhibition for animals treated with letrozole continuously was considerably longer and tumors did not double in volume until 35 weeks.

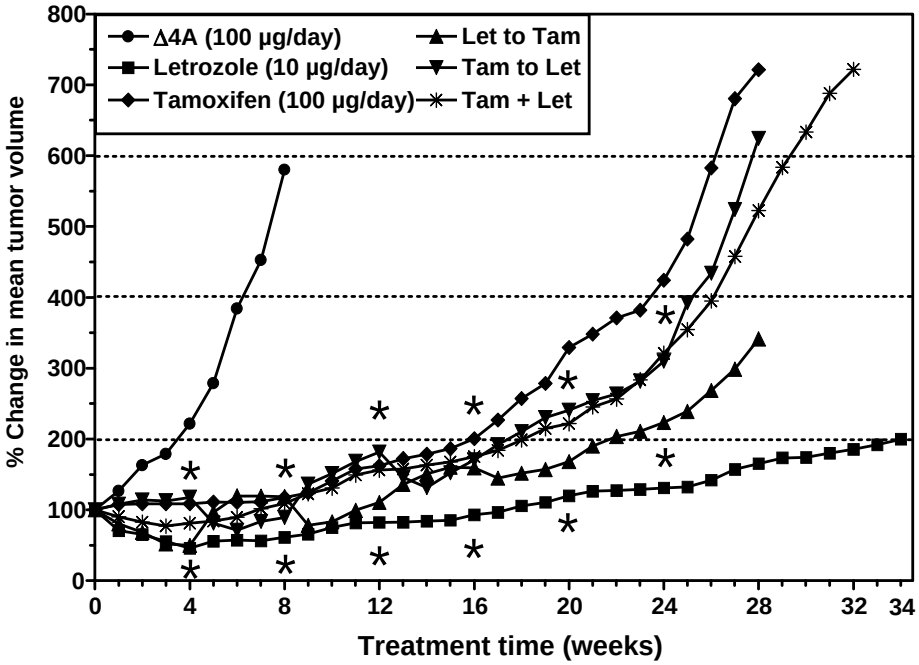

Fig. 4. The effect of alternating treatment with tamoxifen and letrozole on MCF-7Ca tumor doubling time.

4. Discussion

Studies in the tumor model have shown that the aromatase inhibitor, letrozole suppresses tumor growth significantly better than antiestrogens fulvestrant or tamoxifen, a result that has recently been confirmed in breast cancer patients. Although combining the two types of agents to inhibit estrogen synthesis as well as action is an attractive hypothesis, it is apparent from the animal model and confirmed in a clinical trial that the combination of a non-steroidal aromatase inhibitor with tamoxifen does not produce better response rates than these types of aromatase inhibitors alone. In contrast, the combination of the steroidal inhibitor exemestane and tamoxifen may be more effective than either of these two treatments alone. In addition, the possibility of delaying the development of resistance to treatment with letrozole and tamoxifen was not substantiated. Thus, starting treatment with tamoxifen and alternating every 4 weeks with letrozole caused a similar duration of tumor suppression to sequential tamoxifen followed by letrozole. The results suggest that the initial choice of agent may affect the overall duration of response to treatment. The findings may also reflect the longer half-life of tamoxifen and its partial agonist properties when used in the alternating regimen. In conclusion, observations in this preclinical model indicate that letrozole alone may be a more effective treatment than utilizing it in alternating or sequential treatment with tamoxifen.

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