Comparison of the Effects of the Antiestrogens EM-800 and Tamoxifen on the Growth of Human Breast ZR-75-1 Cancer Xenografts in Nude Mice

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ABSTRACT

Although estrone supplementation in ovariectomized (OVX) nude mice bearing ZR-75-1 xenografts caused a 365% increase in average tumor size during the 4-month treatment period, administration of the antiestrogen EM-800 at the daily oral doses of 50, 150, or 400 μg completely prevented estrogen-stimulated tumor growth. At the same doses of tamoxifen, tumor size was inhibited to 189, 117, and 120% above pretreatment values. However, when EM-800 (150 μg/day) was added to the daily 150- and 400-μg doses of tamoxifen, final tumor size was decreased further to 12 and 38% above pretreatment values, respectively. EM-800 (400 μg daily) administered to estrone-supplemented OVX mice caused complete, partial, and stable responses in 11, 22, and 49% of estrone-stimulated tumors, respectively, whereas 19% (7 of 37) progressed. At the same dose of tamoxifen, the corresponding responses were 3% (complete response), 3% (partial response), and 25% (no change), whereas 69% (22 of 32) of tumors progressed. In the absence of estrone supplementation, tamoxifen (400 μg) alone administered to OVX mice stimulated tumor growth to 161% compared with initial size whereas the same dose of EM-800 reduced tumor size by 55%, a value superimposable to that observed in OVX control animals. The agonistic effect of tamoxifen is thus illustrated by the observation that 73% of tumors progressed when tamoxifen was administered alone to OVX animals whereas no tumor progressed with EM-800. The present data strongly suggest that at least part of the initial lack of response and resistance to tamoxifen during tamoxifen treatment in women is due to the estrogenic activity of this compound, whereas the new antiestrogen EM-800 exerts pure antagonistic action.

INTRODUCTION

Breast cancer is the most frequent cancer in women; the odds of developing this cancer are one of nine women during their lifetime. In fact, it is predicted that 181,600 new cases of breast cancer will be diagnosed in the United States in 1997, whereas 44,190 are expected to die from this disease during the same time period (1). Breast cancer has thus become a major medical and public health problem.

Among all factors, estrogens are recognized to play the predominant role in breast cancer development and growth (2—9). Unfortunately, the existing surgical or medical ablative procedures do not permit complete elimination of estrogens in women (10), especially due to the important contribution of the adrenals that secrete high levels of dehydroepiandrosterone and dehydroepiandrosterone-sulfate, which are converted into estrogens in peripheral target tissues (11).

Considerable attention has thus focused on the mechanisms of action of estrogens and especially on the development of blockers of estrogen biosynthesis and action (12—14). Because the first step in the action of estrogens in target tissues is binding to the estrogen receptor (15), a logical approach for the treatment of estrogen-sensitive breast cancer is the use of antiestrogens, compounds that block the interaction of estrogens with their specific receptor.

Tamoxifen has shown important benefits in breast cancer and has become the standard therapy at all stages of the disease. Although 30—50% of the patients with advanced breast cancer show a positive response to tamoxifen, the duration of response is usually limited to 12—18 months with the development of resistance to further treatment with this antiestrogen (5, 6, 16). As demonstrated in a series of studies with human breast cancer cell lines in vitro and in vivo (17—22) and supported by clinical observations (23—28), it seems reasonable to suggest that the loss of positive response to tamoxifen treatment in breast cancer patients could be, at least in part, due to the intrinsic estrogenic activity of the compound. This explanation is supported by the finding that human breast cancer cell lines showing resistance to tamoxifen retain their sensitivity to specific or pure antiestrogens in vitro (22, 29—31) as well as in vivo in nude mice (21, 32, 33).

Because human breast carcinoma xenografts in nude mice are the closest available model of human breast cancer, we have compared the effect of EM-800 and tamoxifen alone and in combination on the growth of ZR-75-1 breast cancer xenografts in nude mice. The present data show that in ovariectomized nude mice supplemented with estrone, EM-800 completely reverses the stimulatory effect of tamoxifen, thus supporting the suggestion that at least part of the initial loss of response and resistance to tamoxifen therapy of breast cancer in women can be due to the estrogenic activity of the compound.

MATERIALS AND METHODS

Human Breast Cancer ZR-75-1 Cells. ZR-75-1 human breast cancer cells obtained from the American Type Culture Collection (Rockville, MD) were routinely cultured in phenol red-free RPMI 1640 (7). The cells were supplemented with 2 mm l-glutamine, 1 mm sodium pyruvate, 100 IU penicillin/ml, 100 μg streptomycin/ml, and 10% (v/v) fetal bovine serum and incubated under an humidified atmosphere of 95% air/5% CO2 at 37°C. Cells were passaged weekly by treatment with 0.05% trypsin/0.02% EDTA (w/v). The ZR-75-1 cells used in the present study were at their 93rd passage at the time of inoculation.

Animals and Tumor Inoculation. Homozygous female HSD nu/nu athymic mice (28—42 days of age) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Mice were housed in vinyl cages equipped with air filter lids that were kept in laminar air flow hoods and maintained under pathogen-limiting conditions. Cages, bedding, food, and water were autoclaved before use. Water was acidified to pH 2.8 and available ad libitum. All animals were ovariectomized before cell inoculation under 2.5% isoflurane anesthesia mixed with oxygen.

ZR-75-1 cells (2.0 × 106) in their logarithmic growth phase were harvested with 0.05% trypsin/0.02% EDTA (w/v) and inoculated s.c. in 0.1 ml of RPMI culture medium containing 30% of Matrigel through a 2.5-cm-long 20-gauge needle in both flanks of OVX2 animals. Tumor growth was initially stimulated by a s.c. implant of E2. E2 implants were prepared in 1-cm-long silastic tubing (inside diameter, 0.062 inch; outside diameter, 0.095 inch) containing 0.5 cm of estradiol:cholesterol (1:10, w/w). Mice bearing tumors of an average diameter of 5.6 ± 0.9 mm (range, 2.2—10.0 mm) were randomly assigned to 12 groups, each containing 15 or 16 mice.

Treatment. On day 0, the E2:chol (1:10, w/w) implants were removed from all animals. Estrone-containing implants (1:25, w/w) were then inserted under isoflurane anesthesia to all animals except for OVX control. EM-800. The present data strongly suggest that at least part of the initial lack of response and resistance to tamoxifen treatment in women is due to the estrogenic activity of this compound.

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2 The abbreviations used are: OVX, ovariectomized; E2, estradiol.
Posteriori pairwise comparisons between group means were performed using the sum of squares. The significance of the treatment effect was tested using the ANOVA. All differences were declared significant at P = 0.05.

RESULTS

Although estrone caused a 365% increase in ZR-75 tumor size during the 4-month treatment period, administration of the oral daily dose of 50, 150-, or 400-μg of the pure antiestrogen EM-800 completely prevented tumor growth (Fig. 2). In fact, at the 400-μg dose, average tumor size was reduced by 25% (P < 0.001) at 4 months compared to the initial size at start of treatment. When the same doses of tamoxifen were administered, it can be seen in the same figure that average tumor sizes were measured at 189, 117, and 120% above pretreatment values at the 50-, 150-, and 400-μg doses, respectively (P < 0.0001 for all doses).

When EM-800 at the daily oral dose of 150 μg was combined with the same dose of tamoxifen, average tumor size decreased from 117% for tamoxifen alone to 12% for tamoxifen + EM-800 (P < 0.0001; Fig. 3A). When the higher dose of tamoxifen was used, i.e., 400 μg daily, the addition of 150 μg of EM-800 decreased tumor size from 120% above average initial size for tamoxifen alone to 38% for tamoxifen + EM-800 (P < 0.01; Fig. 3B). In the presence of EM-800 alone, average tumor size was not significantly different from the pretreatment values.

Although the above-described results were obtained in O VX animals supplemented with estrone, it can be seen in Fig. 4 that the administration of tamoxifen in O VX animals not supplemented with estrone stimulated tumor growth. In fact, after 4 months of treatment, average tumor size was increased to 161 ± 20% (P < 0.001) above pretreatment values, whereas administration of EM-800 led to values superimposable to those obtained in the absence of estrogen in O VX animals receiving the vehicle alone, i.e., 55% below initial tumor size (P < 0.0001; Fig. 4).

It is also of interest to analyze the categories of responses achieved under the experimental conditions described above. In the absence of supplementation with estrone (O VX control), complete, partial, and stable responses were obtained in 21, 33, and 39% of tumors, respectively, whereas only 6% of tumors (2 of 33) progressed (Fig. 5). In O VX animals supplemented with estrone, 100% of tumors (30 of 30) progressed. At the highest dose of EM-800 used, i.e., 400 μg daily, complete, partial, and stable responses were seen in 11, 22, and 49% of tumors, respectively, whereas 19% (7 of 37) progressed. At the same dose of tamoxifen, 3, 3, and 25% of tumors achieved complete,

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**Fig. 1.** Chemical structures of EM-800, EM-652, and tamoxifen.

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EM-800 [(+)-7-pivaloyloxy-3-(4'-pivaloyloxyphenyl)-4-methyl-2(4'+(2'-piperidinoethoxy)phenyl)-2H-benzopyran; Fig. 1] was synthesized in the medicinal chemistry division of the Laboratory of Molecular Endocrinology. Tamoxifen citrate was obtained from Orion Corporation Farmos. All compounds were dissolved in 4% (v/v) ethanol, 4% (v/v) polyethylene glycol 600, 1% (w/v) gelatin, and 0.9% (w/v) NaCl. Animals of the O VX group received p.o. by gavage the vehicle (0.2 ml 4% ethanol, 4% polyethylene glycol 600, 1% gelatin, and 0.9% NaCl) alone whereas the animals of the appropriate groups received daily doses of 50, 150, or 400 μg of EM-800 or tamoxifen alone or in combination as indicated. Tumors were measured once a week with Vernier calipers. Two perpendicular diameters were recorded, and tumor area (cm²) was calculated using the formula: \( \pi \times W/2 \times L/2 \times \pi \) (34). The area measured on the first day of treatment was taken as 100%, and changes in tumor size were expressed as a percentage of initial tumor area. After 126 days of treatment, the animals were sacrificed.

Response Criteria. The criteria of response were as described in Dauvois et al. (34); complete response corresponds to the category of tumors that became undetectable, partial response corresponds to tumors that regressed 50% or more compared with their original size, and stable response is the category of tumors that have regressed less than 50% and have not progressed by 25% or more. Progression is the category of tumors that progressed more than 25%.

Statistical Analysis. The differences in tumor surface areas were analyzed using a univariate ANOVA for repeated measures extracting effects due to treatment, duration of treatment, and time-treatment interaction. Paired measurements of the surface areas (before versus after treatment) were accounted for in the ANOVA model by the addition of a tumor within treatment effect. The significance of the treatment effect was tested using the sum of squares from the tumors within each treatment factor. Other effects were tested using the residual error. Because the assumptions of normality of the residuals and of homoscedasticity were not met, a nonparametric ANOVA was performed on the residual error. Because the assumptions of normality of the residuals and of homoscedasticity were not met, a nonparametric ANOVA was performed on
Fig. 3. Time course of the effect of: A, daily oral doses of 150 µg of EM-800, 150 µg of tamoxifen, or the combination of both drugs; or B, daily oral doses of 150 µg of EM-800, 400 µg of tamoxifen, or the combination of both drugs for 4 months on the average size of ZR-75-1 human breast cancer xenografts in OVX nude mice supplemented with an implant of estrone. OVX nude mice receiving the vehicle alone or supplemented with estrone implants are added as controls. The size of tumors at start of treatment was 31.1 ± 0.8 mm². Results are expressed as percentage of pretreatment values (means of 25–37 tumors/group; bars, SE).

DISCUSSION

The present data clearly show, under in vivo conditions in nude mice, the stimulatory effect of tamoxifen on the growth of human breast cancer xenografts, whereas the novel antiestrogen EM-800 has no stimulatory effect. In fact, 73% of tumors progressed when tamoxifen was administered to OVX animals, whereas no tumor progressed with EM-800. Moreover, in OVX animals supplemented with estrone, EM-800 (150 µg daily) could completely neutralize the increase in partial, and stable responses, respectively, whereas 69% (22 of 32) progressed.

The addition of EM-800 (150 µg) to tamoxifen reduced the percentage of progressing tumors from 71 to 24% and from 69 to 32% at the daily 150- and 400-µg doses of tamoxifen, respectively. The agonistic effect of tamoxifen is particularly well illustrated by the observation that 73% of tumors progressed when tamoxifen alone was administered to OVX animals, whereas no tumor progressed with EM-800 and only 6% progressed in OVX mice receiving the control vehicle.
average tumor size observed with tamoxifen at both the 150-μg (Fig. 3A) and 400 μg (Fig. 3B) doses. The present demonstration of a stimulatory effect of tamoxifen on human breast cancer growth is in agreement with previous data obtained in human breast cancer cell lines in vitro (17–19, 22) as well as in vivo in nude mice (20, 21). The present experimental data are also in agreement with clinical observations suggesting the stimulatory effect of tamoxifen on breast cancer in women (23–28). Particularly convincing evidence of the estrogenic activity of tamoxifen is also provided by the finding that human breast cancer cell lines showing resistance to tamoxifen retain their sensitivity to specific or pure antiestrogens in vitro (22, 29–31) as well as in vivo in nude mice (21, 32, 33).

In the present model, EM-800 was administered to OVX mice supplemented with estrone to provide a constant source of estrogens and to avoid the compensatory increase in ovarian estrogen secretion that occurs when the compound is administered to otherwise untreated intact animals. Because of its pure antiestrogenic activity, EM-800 administered to intact mice causes an increase in gonadotropin secretion by the anterior pituitary gland, which leads to increased ovarian estrogen secretion. This phenomenon results from inhibition of the negative feedback action of estrogens at the hypothalamic level. The particularly large size of the tumors at start of treatment (31 ± 0.8 mm³) probably explains the lack of complete reversal of the effect of estrone by EM-800 as observed in other studies (36).

Although adjuvant treatment with tamoxifen delays breast cancer recurrence and improves survival in early breast cancer and induces remission in patients with advanced disease, its benefits are frequently limited by the development of tamoxifen resistance (37). Similarly, in the in vivo model using nude mice, tamoxifen inhibited MCF-7 tumor growth for 4–6 months, but tumor growth then continued despite tamoxifen treatment (33, 38). In analogy with the present data, Gottardis et al. (20) have observed the acquired ability of tamoxifen to stimulate rather than to inhibit tumor growth. Because, as mentioned above, pure antiestrogens can inhibit the stimulatory effect of tamoxifen (21, 32, 37), such data suggest that the stimulatory effect of tamoxifen upon long-term treatment is due to the intrinsic estrogenic activity of the compound or its metabolites (39).

Treatment of nude mice bearing MCF-7 xenografts with 10 mg of ICI 182780 once a week led to a transient decrease of tumor size, followed by a plateau of no change for about 200 days, followed by progression (21). In mice treated with ICI 182780, regrowth of tumors or resistance to ICI 182780 occurred in most tumors (21).

It is of interest to mention that, of all of the compounds tested, the novel nonsteroidal pro-drug EM-800 and its active metabolite EM-652 exert the most potent antagonistic effects on estradiol-induced proliferation in T-47D, ZR-75-1, and MCF-7 human breast cancer cells in culture (9). Furthermore, the absence of a stimulatory effect on basal cell proliferation in the three estrogen-sensitive human breast cancer cell lines used shows that EM-652 and EM-800 are pure antiestrogens devoid of partial agonist activity in human breast cancer tissue. It was also of interest to observe that the antiestrogenic activity of EM-652 and EM-800 on E₂-induced cell proliferation in T-47D cells is at least two orders of magnitude more potent than tamoxifen, 2.5- to 3.6-fold more potent than OH-tamoxifen, and 3.8-, 2.7-, and 16.3-fold more potent than OH-toremifene, ICI-182780, and ICI-164384, respectively. On the other hand, EM-800 was 46-fold more potent than Droloxifene in inhibiting E₂-induced T-47D cell proliferation. As mentioned above, EM-800 and EM-652 have no estrogenic activity in the three breast cancer cell lines studied, whereas OH-tamoxifen, Droloxifene, and Toremifene cause a significant stimulation of ZR-75-1 and MCF-7 human breast cancer cell proliferation (9).

The stimulatory effect of tamoxifen or OH-tamoxifen on human breast cancer cell growth has been reported previously by many laboratories under in vitro (2, 17, 18, 40–48) as well as in vivo (20) conditions. Such intrinsic estrogenic activity of tamoxifen is likely to limit its success in the treatment of breast cancer in women (49). In addition to the data mentioned earlier, the estrogogenic action of tamoxifen in breast cancer in women is supported clinically by the tumor flare observed at start of therapy (50–52). This early stimulatory effect of tamoxifen is analogous to the present data showing a stimulatory effect of the same drug on the growth of ZR-75-1 xenografts. The withdrawal response observed following arrest of tamoxifen in patients who progress under tamoxifen therapy (24, 25) can also result from the estrogogenic activity of tamoxifen. The resistance that develops during long-term treatment with tamoxifen could involve other mechanisms, such as changes in metabolism.

It seems reasonable to expect that the availability of a pure antiestrogen, in addition to avoiding the risk of inducing endometrial carcinoma (53), should show significant benefits over tamoxifen in the treatment of breast cancer. In fact, due to the unsatisfactory characteristics of the drugs available, only partial blockade of estrogens could thus far be achieved in women suffering from breast cancer, whereas the role of estrogens in this disease is unlikely to have been satisfactorily evaluated. In view of the particularly high potency of this new antiestrogen and its highly specific antiestrogenic characteristics illustrated in estrogen-sensitive human breast cancer cells both in vitro (9) and in vivo (this report), it is hoped that achieving a more complete blockade of the action of estrogens could result in a more rapid, more complete, and longer-lasting inhibition of breast cancer growth.

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