ABSTRACT

Human breast cancer proliferates as heterogeneous cell populations that exhibit different sensitivities to therapeutic agents. A logical approach to control these different cancer cell populations is the use of combined treatment with agents that block cell proliferation or induce apoptosis via different mechanisms. We therefore investigated the effect of treatment with the novel pure antiestrogen EM-800, alone or in combination with chemotherapy, on the growth of ZR-75-1 human breast tumors in nude mice, a well-recognized model of human breast cancer. Mice bearing estrone-releasing silastic implants as estrogenic stimulus received EM-800 or cyclophosphamide alone or in combination for 227 days. Cyclophosphamide (256 mg/kg/2 weeks) was administered by i.p. injection in 64 mg/kg fractions over 4 consecutive days with repetition of the cycle every 14 days. EM-800 was administered p.o. once daily at the maximally effective dose of 300 μg/mouse. After 227 days of treatment, average tumor size in mice receiving estrone alone was 192% higher than pretreatment. The average tumor size of mice treated with chemotherapy was reduced by 47%, whereas on the other hand, EM-800 caused a 81% decrease of the value of the same parameter. The combined treatment (EM-800 + cyclophosphamide), on the other hand, resulted in a 95% decrease in tumor size compared with control estrone alone. In fact, EM-800 alone decreased tumor size to 55% of the value at the start of treatment, whereas the addition of cyclophosphamide to the antiestrogen further decreased tumor size to as low as 15% of the pretreatment value. The combination of EM-800 and cyclophosphamide resulted in 95% of complete or partial responses compared with 61 and 27% with EM-800 and cyclophosphamide alone, respectively. In fact, in the combination therapy group, only one tumor remained stable, while 17 regressed >50% and four disappeared. It is noteworthy that no tumor progressed with EM-800 alone or in combination with cyclophosphamide. The present data show, for the first time, that the addition of cyclophosphamide to a pure antiestrogen used at a maximal dose causes a more potent inhibition of human breast tumor growth, thus suggesting that combined treatment using a maximal dose of a pure antiestrogen and a chemotherapeutic agent(s), two classes of compounds having different mechanisms of action, could further improve breast cancer therapy above the results achieved with a potent and pure antiestrogen alone in estrogen-sensitive breast cancer.

INTRODUCTION

Breast cancer is the most frequent cancer in women, affecting one of every nine women during their lifetime. In fact, it is predicted that 175,000 new cases of breast cancer will be diagnosed in the United States in 1999, whereas 43,300 women are expected to die from this disease during the same time period (1). Breast cancer is thus recognized as 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), and it is well known that estrogen deprivation causes regression of breast tumors. Because the first step and an essential common pathway in the action of estrogens in target tissues is binding to the estrogen receptors (3, 4), a logical approach for the treatment of estrogen-sensitive breast cancer is the use of antiestrogens that competitively bind to estrogen receptors and block estrogen action.

Tamoxifen has been widely used for the treatment of breast cancer over the past decades and has shown important benefits in breast cancer therapy. Unfortunately, in patients who initially respond, recurrence of the cancer during or after treatment is observed in most cases. The absence or loss of response to Tamoxifen can possibly be attributed to a suboptimal blockade of estrogen action (5, 6), which could be explained by the lack of inhibition by Tamoxifen of the activation of the estrogen receptor by growth factors and other factors that act through the Ras-mitogen-activated protein kinase pathway at AF-1 of both estrogen receptors α and β (7–9). Because of these limitations of Tamoxifen, major efforts have focused on the development of pure antiestrogens devoid of intrinsic agonist activity (10–12) and the ability to block both the AF-1 and AF-2 functions of the estrogen receptors (9, 13).

The pure nonsteroidal antiestrogen EM-800 is the most potent of the known antiestrogens (13, 14). EM-652, the active metabolite of EM-800, displays the highest known affinity for the estrogen receptor (15) and acts as a pure antiestrogen by blocking the estrogenic action of 17β-estradiol mediated by estrogen receptors α and β at both the AF-1 and AF-2 sites (9). In contrast to a series of other nonsteroidal antiestrogens, EM-800 inhibits estrogen-induced alkaline phosphatase activity in endometrial cells as well as mammary carcinoma cell proliferation in vitro without any agonist activity (14, 16). In addition, EM-800 is active p.o. and produces a maximal inhibition of estrogen-stimulated ZR-75-1 tumor growth, without evidence of escape during long-term treatment (17).

Our initial hypothesis was that a treatment combining both hormonal and nonhormonal therapies at the outset could possibly increase the rate of recurrence-free survival by killing tumor cell populations with varying degrees of estrogen sensitivity by inducing cell death by multiple pathways. Combination treatment could also potentially allow the use of lower doses of chemotherapy, thereby resulting in a decrease in side effects. Because human breast carcinoma xenografts in nude mice are the closest available model of human breast cancer (18), we have compared the effects of the pure antiestrogen EM-800 and cyclophosphamide, a chemotherapeutic agent, either alone or in combination, on the growth of the well-characterized, estrogen-sensitive ZR-75-1 breast cancer cells inoculated in OVX nude mice supplemented with E1-releasing silastic implants.

Effects of the Antiestrogen EM-800 (SCH 57050) and Cyclophosphamide Alone and in Combination on Growth of Human ZR-75-1 Breast Cancer Xenografts in Nude Mice

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3 The abbreviations used are: EM-800, (5S,8S)-[4-(7-(2,2-dimethyl-1-oxopropoxy-4-methyl-2-[4-2-(1-piperidinyl) ethoxy]phenyl)-2H-1-benzopyran-3-yl[phenyl]-2,2-dimethylpropanoate; OVX, ovariectomized; E1, estrone.

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EM-800 AND CYCLOPHOSPHAMIDE EFFECTS ON ZR-75-1 TUMORS

MATERIALS AND METHODS

Human ZR-75-1 Breast Cancer Cells. ZR-75-1 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in phenol red-free RPMI 1640 (19). The cells were supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 100 IU penicillin/ml, 100 μg of 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 and were harvested in their logarithmic phase. The ZR-75-1 cells used in the present study were at their 93rd passage at the time of inoculation.

Animals and Tumor Inoculation. Female homozygous HSD nu/nu athymic mice (28–42 days of age) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The mice (five per cage) were housed in vinyl cages equipped with air filter lids, which were kept in laminar air flow hoods and maintained under pathogen-limiting conditions. The photoperiod was 14 h of light and 10 h of darkness (lights on at 7 a.m.). Cages, bedding, and food (Agway Pro-Lab R-M-H Diet #4018) were autoclaved before use. Water was acidified to pH 2.8, autoclaved, and provided ad libitum. The mice were OVX under 2.5% (v/v) isoflurane-induced anesthesia 1 week before cell inoculation. At the time of ovariectomy, an implant of estradiol (E2) was inserted s.c. to stimulate initial tumor growth. 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 a 1:10 (w/w) mixture of estradiol and cholesterol. One week after ovariectomy, 2.0 × 107 ZR-75-1 cells were inoculated s.c. in 0.1 ml of RPMI 1640 containing 30% (v/v) Matrigel on both flanks of each mouse through a 2.5-cm-long, 20-gauge needle. Four weeks after cell inoculation, the E2 implants were replaced by E2-containing implants (E2:cholesterol, 1:25, w/w).

Treatments. One day prior to initiation of treatments, 60 mice bearing ZR-75-1 tumors of an average area of 44.1 ± 1.6 mm² (range, 5.7 to 95.6 mm²) were randomly assigned to five groups (with respect to tumor size), each containing 12 mice (23 or 24 tumors). At this time, E2 implants were removed from the animals in the OVX control group (OVX). Estrone-containing implants in the four other groups were changed every 6 weeks thereafter. The average body weight measured at the start of treatment was used to calculate the doses of EM-800 and cyclophosphamide for the duration of the experiment. EM-800 was synthesized in the medicinal chemistry division of the Laboratory of Molecular Endocrinology (13). Animals dosed with EM-800 alone or in combination with cyclophosphamide received oral daily doses of 300 μg of EM-800 (12 mg/kg, on average) suspended in 0.2 ml of 0.4% (w/v) methylcellulose, whereas the animals in the three other groups received 0.2 ml of the vehicle alone. Cyclophosphamide (Procystox) was purchased from Carter-Horner Inc. and dissolved in saline solution. Cyclophosphamide solutions were prepared freshly, used for two cycles, and administered by i.p. injection at the dose of 64 mg/kg (1.6 mg in a volume of 0.2 ml/mouse) once daily for 4 consecutive days. Cyclophosphamide treatment cycles were repeated every 2 weeks for a total of 256 mg/kg/2 weeks (6.4 mg/mouse/2 weeks). This dose, which corresponds to the best efficacy:toxicity ratio, was selected on the basis of preliminary tests (data not shown) and on doses used in a previous study (18).

Tumor Measurements and Necropsy. Two perpendicular diameters were recorded, and tumor area (mm²) was calculated using the formula: \( A = \frac{L \times W}{2} \times \pi \) (20). The area measured on the first day of treatment was taken as 100%, and changes in tumor size were expressed as percentage of the initial tumor area. Because of several mortalities in the group treated with cyclophosphamide alone during the last 2 weeks of the experiment, mean tumor size as well as the categories of response achieved after treatment were analyzed using the tumor area data collected on day 227 for all groups. The number of animals in the various groups on day 227 were: OVX control = 11; OVX + E1 = 9; OVX + E1 + EM-800 = 11; OVX + E1 + cyclo = 7; and OVX + E1 + EM-800 + cyclo = 9.

After 241 days of treatment, the remaining animals were anesthetized with isoflurane and killed by cervical dislocation. To characterize the effect of estrogen and antiestrogen on the mice, an estrogen-responsive tissue, the uterus, was immediately removed, freed from connective and adipose tissue, and weighed.

Response Criteria. The response criteria were adapted from Dauvois et al. (21). Tumor response was assessed at the end of the study or at the death of each mouse, if it occurred during the course of the experiment. In this case, only data of mice that survived for at least half of the study (115 days) were used in the tumor response analysis. Complete regression identifies those tumors that were undetectable at the end of the experiment; partial regression corresponds to the tumors that regressed ≥50% of their original size; stable response refers to tumors that regressed <50% or progressed ≤50%; and progression refers to tumors that progressed >50% compared with their original size.

Statistical Analysis. The variations of the total surface areas of tumors between day 1 and day 227 were analyzed using a two-way ANOVA for repeated measurements. The treatment effect is thus considered completely confounded with the differences between the groups of animals used within each modality of treatment and is therefore tested against the error term estimated for the animals within the groups. A posteriori pairwise comparisons were also performed using least square means statistics.

An effect of the five different modalities of treatment on the uterine weight as well as on the final body weight was assessed using a one-way ANOVA. A posteriori pairwise comparisons were performed using least square means statistics.

The significance of difference was accepted for \( \alpha < 0.05 \). All statistical tests were performed using the SAS software (SAS Institute, Cary, NC).

RESULTS

Effects of EM-800 and Cyclophosphamide on ZR-75-1 Tumor Growth. Supplementation with E1 alone (OVX + E1) caused a 192% increase in ZR-75-1 tumor size during the 8-month treatment period (Fig. 1). Administration of the pure antiestrogen EM-800 at the daily oral dose of 300 μg completely prevented tumor growth. In fact, average tumor size in this group was 55% lower compared with the initial value at start of treatment (\( P < 0.001 \)). The value thus achieved was not significantly different from that observed after ovariectomy.
alone (OVX), where tumor size decreased by 68% below initial tumor size ($P < 0.001$, not significant between OVX and OVX + E$_1$ + EM-800).

Treatment with cyclophosphamide at the dose of 256 mg/kg every 2 weeks caused a rapid decrease in ZR-75-1 tumor size for ~21 days, an effect that was comparable with that seen with EM-800 alone during this early period. However, despite repeated cycles of cyclophosphamide, average tumor size in cyclophosphamide-treated mice remained approximately stable from days 22 to 120 and then increased progressively to reach 155% of initial tumor size on day 227, a value not statistically significantly different from that on day 0. The average tumor size measured at the end of treatment with cyclophosphamide was lower than that of E$_1$-treated mice, although not statistically significantly different. This value, however, was significantly higher than that of the OVX control group, the EM-800-treated group, and the combined treatment group ($P < 0.001$ versus all three groups).

Combination treatment with EM-800 and cyclophosphamide resulted in a more rapid and more important decrease in tumor size than either treatment alone. In fact, average tumor size was 85% ($P < 0.001$) lower than initial size after 227 days of combined treatment. The average tumor size in mice treated with both EM-800 and cyclophosphamide was thus 67 ± 3.8% ($P < 0.01$) lower than that of mice treated with EM-800 alone (15 ± 3.3% versus 45 ± 5.0% of original size in favor of combined treatment) and 53 ± 9.8% ($P < 0.05$) lower than that of OVX mice (15 ± 3.3% versus 32 ± 6.4% of original value in favor of combined treatment).

**Category of Response.** In addition to the effect on tumor size, the category of response observed for each individual tumor at the end of the experiment is an important parameter of treatment efficacy. In OVX mice, complete response or disappearance of tumor was achieved in 27% of all tumors. A partial response or a >50% decrease in tumor size was observed in 45% of tumors, whereas 27% of tumors remained stable (decrease in single progression < 50% or progression less than 50%) respectively. In fact, none of the tumors progressed. In OVX animals supplemented with E$_1$, 67% of tumors progressed, 22% remained stable, 11% regressed partially, and no tumor reached the complete regression category (Fig. 2). In the EM-800-treated group, complete, partial, and stable responses were seen in 4, 57, and 39% of tumors, respectively. There were no progressions tumors after 227 days of treatment with EM-800 alone. Cyclophosphamide alone did not result in any complete response, whereas 45% (10 of 22) of tumors progressed and 27% of tumors were classified in each of the partial and stable response categories. Combined treatment with cyclophosphamide and EM-800 resulted in a greater proportion of complete and partial regressions than ovarie-tomy or either monotherapy. In fact, in mice that received combination therapy for 227 days, 18% (4 of 22) of the tumors disappeared, whereas 77% of the tumors regressed >50% (17 of 22), and only one tumor remained in the stable category (50%) while no tumor progressed. It is interesting to note that no progressing tumors were observed in the groups that received EM-800, either alone or in combination with cyclophosphamide, as well as in the OVX control group.

**Body Weight.** The mean body weight (corrected for tumor weight) of mice in the E$_1$-supplemented control group was not significantly different from that observed in the OVX control group as well as in the EM-800-treated group (Table 1). On the other hand, the average body weights of mice receiving cyclophosphamide, alone or in combination with EM-800, were 14 and 17% lower than that of E$_1$-supplemented control mice ($P = 0.015$ and $P < 0.01$, respectively).

**Uterine Weight.** Treatment with 300 µg of EM-800 daily, alone or in combination with cyclophosphamide, resulted in a complete blockade of the stimulatory effect of E$_1$ on uterine weight (Table 1). In fact, the mean uterine weight of OVX control mice (1.9 ± 0.3 mg/g of body weight) was not significantly different from that of estrogen-supplemented mice receiving EM-800 alone (1.7 ± 0.1 mg/g of body weight) or the combined therapy (1.6 ± 0.1 mg/g of body weight). On the other hand, the average uterine weight of mice treated with E$_1$ alone increased to 11.8 ± 0.9 mg/g of body weight, whereas treatment with cyclophosphamide alone decreased this value to 8.3 ± 1.0 mg/g of body weight.

**DISCUSSION**

The present data show that the addition of cyclophosphamide to the pure antiestrogen EM-800 leads to a more potent inhibition of growth of human breast tumor xenografts in nude mice than achieved with each compound alone. In fact, although EM-800 alone causes a major inhibitory effect, i.e., an 85% inhibition of tumor size relative to the
control OVX E\textsubscript{2}-supplemented mice, a further 10\% inhibition is achieved when cyclophosphamide is added to EM-800 to reach a 95\% inhibition. Cyclophosphamide alone, on the other hand, inhibits by only 47\%, an apparent resistance developing during treatment.

Multiple mechanisms have been suggested for the antiestrogen-induced growth inhibition of estrogen receptor-positive cell lines. Antiestrogens increase apoptotic activity (22, 23), down-regulate the levels of the antiapoptotic protein bcl-2 (24), and cause a decrease in growth rate secondary to a block in the G\textsubscript{1}/G\textsubscript{2} phase of the cell cycle (14, 22, 25, 26). On the other hand, the cytotoxic effect of cyclophosphamide is predominantly attributable to the transfer of its alkyl group to DNA, which ultimately causes DNA double-strand breaks. Cells that are unable to repair this DNA injury undergo apoptosis (27). Although cyclophosphamide is not a cell cycle-specific agent, cells are most vulnerable to alkylation in the late G\textsubscript{1} and S phases of the cell cycle, and replicating cells are thus more likely to be affected by this mechanism (27).

The rate of disease recurrence with metastatic spread remains high, despite the use of treatment modalities such as surgery, radiation therapy, chemotherapy, and current hormonal manipulation (28). A potential limitation of endocrine therapy is that some hormone-sensitive cells could survive in a resting state upon hormone deprivation as opposed to those that undergo apoptosis (29). This limitation might possibly be overcome by longer term endocrine treatment, as found recently with combined androgen blockade in prostate cancer (30). It could also be postulated that the eventual loss of positive response to endocrine therapy could be, at least in part, due to heterogeneous cell populations that respond differently to therapeutic agents (31). It thus seems reasonable to suggest that the combined use of therapeutic agents that induce apoptosis via different mechanisms can potentially lead to a greater and more rapid inhibition of breast tumor growth and increase apoptosis or even tumor eradication. In a large proportion of tumors, the efficacy of combined treatment with chemotherapy and radiation therapy has been demonstrated in other tumor models containing heterogeneous cell populations (32).

EM-800, in keeping with the properties of a pure antiestrogen, completely blocked the stimulatory effect of E\textsubscript{2} on ZR-75-1 tumor and uterine weight in nude mice. The weight of these tissues was reduced to DNA, which ultimately causes DNA double-strand breaks. Cells that are unable to repair this DNA injury undergo apoptosis (27). Although cyclophosphamide is not a cell cycle-specific agent, cells are more vulnerable to alkylation in the late G\textsubscript{1} and S phases of the cell cycle, and replicating cells are thus more likely to be affected by this mechanism (27).

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EM-800, in keeping with the properties of a pure antiestrogen, completely blocked the stimulatory effect of E\textsubscript{2} on ZR-75-1 tumor and uterine weight in nude mice. The weight of these tissues was reduced to that achieved by ovariectomy alone, which corresponds to the limit expected from complete blockade of estrogen action achieved with EM-800. The particular blockade of estrogen action achieved with EM-800 is of interest of this study is that, for the first time, a pure antiestrogen is used in combination with a nonhormonal chemotherapeutic agent, and the pure antiestrogen is used at a maximally effective dose. All previous studies have used Tamoxifen, a mixed agonist/antagonist compound that does not block the AF-1 site of the estrogen receptor (9).

The present in vivo study using a pure estrogen antagonist in a nude mouse model of estrogen-responsive human breast cancer clearly indicates the benefits of adding cyclophosphamide to a complete blockade of estrogen action achieved with EM-800. The particular interest of this study is that, for the first time, a pure antiestrogen is used in combination with a nonhormonal chemotherapeutic agent, and the pure antiestrogen is used at a maximally effective dose. All previous studies have used Tamoxifen, a mixed agonist/antagonist compound that does not block the AF-1 site of the estrogen receptor (9).

The present data show that the addition of cyclophosphamide decreased tumor size from a 85\% inhibition by EM-800 alone to a 95\% or near complete inhibition when both drugs are combined. Considering the low level of side effects observed thus far with the pure antiestrogen and the more important secondary effects of chemotherapy, it would be important to perform clinical trials in both the pre (neoadjuvant) and post (adjuvant) setting to determine with precision the potential advantages of combining a pure antiestrogen and chemotherapy, possibly at a lower dose. As mentioned above, pure antiestrogens are likely to provide results quite different from those obtained with Tamoxifen, and only appropriately designed clinical trials will provide the required answer.

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