Effect of Combined Treatment with the Pure Antiestrogen EM-800 and Radiotherapy on the Growth of Human ZR-75-1 Breast Cancer Xenografts in Nude Mice

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ABSTRACT

Human breast tumors are usually composed of heterogeneous cell populations that exhibit different sensitivities to therapeutic agents. We therefore investigated the effect of treatment with various regimens of the novel pure antiestrogen EM-800, alone or in combination with external beam radiation therapy, on the growth of human ZR-75-1 xenografts in athymic mice. The animals received a maximal dose of EM-800 (300 µg, p.o.) and/or radiotherapy at the dose of 10 Gy. 2.5 Gy fractions were administered over a 9-day period in four sessions of 13.7 min each (250-kilovolt Siemens with 2-mm aluminum filtration at 90 cm from the source origin). EM-800 was administered p.o. once daily, whereas radiotherapy was repeated every 35 days. Tumor size was expressed as a percentage of the initial tumor size, which was assigned a value of 100%. Average tumor size increased by 514% in ovariectomized mice supplemented with estradiol alone for 259 days compared with the pretreatment value. Treatment with radiotherapy or EM-800 alone resulted in 11 and 73% decreases in mean tumor size, respectively, whereas combined treatment given simultaneously at the beginning caused a dramatic 98% decrease in tumor size. The start of radiotherapy on day 35 in EM-800-treated mice, or conversely, the start of EM-800 in irradiated mice at the 35-day time interval, resulted in somewhat lower, 88% and 95%, decreases in tumor size, respectively. In animals receiving EM-800 alone, 40% of tumors disappeared, thus indicating a cytotoxic effect caused by the estrogen blockade achieved with the pure antiestrogen. Eighty-six percent of the original tumors disappeared under continuous combined treatment. Most importantly, no tumor reappeared under estrogenic stimulation after stopping treatment, thus indicating cure of 86% of the tumors in the group of animals who received the combination therapy. The present data indicate that combined treatment with EM-800 and radiotherapy yields a faster response, a greater decrease in tumor size, and a higher percentage of complete responses or tumor disappearance (cure) than either treatment used alone. The present data also suggest that maximal benefits are achieved when the pure antiestrogen is administered continuously, starting at the same time as radiation therapy and continued without interruption as adjuvant therapy. The present data also clearly show that efficient blockade of estrogens with a potent and pure antiestrogen is not only cytostatic but is cytotoxic and can lead to the disappearance of an important proportion of tumors or cure.

INTRODUCTION

Breast cancer is the most frequent cancer in women in the United States, accounting for 30% of all cancers (1). In fact, it is estimated that 175,000 new cases of breast cancer will be diagnosed and 43,300 women will die from this disease in the United States in 1999. Despite the availability of surgery, radiation therapy, chemotherapy, and hormone therapy, the rate of recurrence with metastases after first treatment of breast cancer remains high, possibly due in part to the heterogeneity of cancer cell populations that respond differently to each therapy (2).

Because estrogens are known to play a major role as stimulators of breast cancer development and growth (3), blockade of estrogen action by pure antiestrogens is a logical approach (4–6), especially using a third-generation selective estrogen receptor modulator such as EM-800 that possesses pure and highly potent antiestrogenic activity in the human mammary gland and endometrium (7–11). EM-800 has also been shown to exert beneficial effects in women who had failed Tamoxifen (12).

Radiotherapy, on the other hand, is well known for its beneficial effect in breast cancer, especially as an adjuvant to surgery. In a recent report of the American Radiation Therapy Oncology Group, androgen deprivation combined with radiotherapy resulted in a marked increase of local tumor control and disease-free survival compared with irradiation alone in patients with locally advanced prostate cancer (13). It thus seems reasonable that the combined use of therapeutic agents that block cell proliferation or induce apoptosis via different mechanisms can potentially lead to greater inhibition of breast cancer growth, as demonstrated in other tumor models with the combination of chemotherapy and radiation therapy (14) as well as the association of Tamoxifen and chemotherapy in human breast cancer (15). It is also well recognized that an inverse relationship exists between tumor volume and tumor control by irradiation, with smaller tumors requiring lower doses of radiation for their eradication (16). The present study examines the effect of the novel pure antiestrogen EM-800, radiotherapy, or a combination of the two approaches on the growth of the well-characterized estrogen-sensitive ZR-75-1 human breast tumors in nude mice. Various schedules of administration were also examined.

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 (17). 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 a humidified atmosphere of 95% air/5% CO₂ at 37°C. Cells were passaged weekly by treatment with 0.083% trypsin/0.3 mM EDTA. The ZR-75-1 cells used in the present study were at their 89th 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). Mice were housed five per vinyl cage equipped with air filter lids and kept in laminar air flow hoods under pathogen-limiting conditions. The photoperiod was 14 h of light and 10 h of dark (lights on at 07:00 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. Bilateral ovariectomy was performed in all animals one week before cell inoculation under 2.5% isoflurane anesthesia mixed with oxygen. At the same time, an implant of E₂³ was inserted s.c. to stimulate initial tumor growth and appearance. E₂ implants were prepared in 1-cm long silastic tubing.
EFFECT OF EM-800 AND RADIATION THERAPY

Tumor Measurement. Two perpendicular diameters were recorded, and tumor area (mm²) was calculated using the formula: \( \frac{L \times W^2}{\pi} \) (18). The area measured on the first day of treatment was taken as 100%, and changes in tumor size were expressed as percentage of initial tumor area.

Necropsy and Histopathology Analysis. After 259 days of treatment, the animals were euthanized with isoflurane and killed by cervical dislocation. To quantitate the effect of the estrogen and antiestrogen on a classical estrogen-sensitive parameter, uteri were immediately removed, freed from connective and adipose tissue, and weighed. To assess the effect of radiation on certain tissues surrounding the tumor, a single hepatic section was collected in the left lateral lobe. Additionally, two sections of the skin overlying the xenografts in the flank region were collected in all animals. These collected tissues were fixed, stained in paraffin, cut in 4-μm-thick sections, and stained with H&E.

Response Criteria. The criteria of response were adapted from Dauvois et al. (19). In brief, complete regression identifies those tumors that were undetectable for at least 3 weeks during treatment, partial regression corresponds to tumors that regressed ≥50% of their original size, stable response refers to tumors that regressed <50% or progressed ≤50%, and progression indicates tumors that progressed >50% compared with their original size. Mice treated for at least 70 days were included in the analysis.

Experiment 1. One hundred twenty-four mice bearing ZR-75-1 tumors of a largest diameter >2.5 mm were divided into seven groups (with respect to tumor size), each containing 15–20 mice at the start of treatment. The animals in the control groups (groups A and B in Fig. 1) received the oral vehicle [0.4% (w/v) methylcellulose] alone, whereas the animals in the three other groups received daily oral doses of 300 μg of EM-800, radiation therapy 10 Gy/35 days, or a combination of both treatments, respectively, for an additional period of 120 days. As for the first experiment, mice that were not treated with the oral antiestrogen EM-800 received the vehicle alone.

EM-800 Therapy. EM-800 (5 μg) was given once daily. The 300-μg dose of EM-800 was 0.67 mm² (range, 6 to 53 mm²; Ref. 18) were randomly assigned to 12 groups, each containing 10–20 mice. On day 0 of the experiment, E₁-releasing implants were removed from animals in the OVX control group. OVX animals first received the most potent natural estrogen to initiate cell proliferation and the development of tumor. Thereafter, the E₁ implants were replaced by E₂ as a model of postmenopausal women when E₁ is the main circulating estrogen and is converted into E₂ in peripheral tissue.

RADIATION THERAPY.

To protect the pathogen-limited environment, we developed a method that permits us to limit the radiation therapy exposure to the tumors of 12 mice simultaneously (Fig. 2). Mice remained in their vinyl microisolators during transport to maintain the pathogen-limiting conditions. Temperature and humidity were controlled during radiation. A portable HEPA filtration system (Biobubble, Fort Collins, CO) was installed in the radiation therapy room at least 3 h before the start of each radiation therapy session. Mice were anesthetized directly in their microisolators under 3.5% isoflurane mixed with oxygen. Handling of mice was performed under positive air flow pressure (HEPA filtration) in a modified baby isolator (Isolette) sterilized and adjusted for the specific needs of the experiment. In this controlled environment, mice were transferred to the irradiation Plexiglas box specially designed to maintain anesthesia under isoflurane through connection to a portable anesthesia unit. Because the working environment has natural air circulation caused by the positive air flow pressure, the concentration of isoflurane and its flow were increased to 4% and 4 l/min, respectively, in the box during manipulation. The box contains 12 circular holes of 2.5 cm diameter each to avoid absorption of radiation by the Plexiglas.

Twelve anesthetized mice were thus immobilized with sterile medical adhesive tape in the box with tumors directly facing each of the 12 holes. The cage was then closed hermetically (for sterility and anesthesia), and the isoflurane flow was reduced for maintenance of anesthesia (1.5% isoflurane and 0.7 l/min). The Isolette was opened in front of the Biobubble, and the box was mounted on a vise behind a lead plate containing holes of design, location, and dimension identical to those of the Plexiglas box. From the lead plate holes, only the tumor of each mouse protruded. Radiotherapy was administered in four fractions of 2.5 Gy for 13.7 min for a total of 10 Gy over a 9 day-period using a 250-kilovolt Siemens with 2 mm aluminum filtration at 90 cm from the source origin. During each treatment, the microisolator was ventilated using a HEPA-filtered air flow from the Biobubble to eliminate the residual isoflurane. After each treatment, awakening of mice was controlled, and they were then transferred to their initial cages. The radiotherapy course of 10 Gy in four fractions was repeated every 35 days. These radiation parameters were established by an oncological physicist of the radio-oncology service of the Hôpital-Dieu Hospital to deliver a homogeneous radiation dose to each individual tumor. To appreciate the possible interaction between EM-800 and radiotherapy, the dose of radiation therapy was not established to obtain a maximal effect but to provide stabilization of tumor growth.

Evaluation of “Cure.” A large number of tumors disappeared during the course of the study. To evaluate the cure rate of each therapeutic modality, mice with tumors that regressed completely for 3 weeks were treated with estradiol as shown in Fig. 3. Tumors that did not reappear after 120 days of estrogenic stimulation were considered “cured.” These mice were evaluated up to 180 days after addition of an E₁ implant.

Statistical Analysis. The variations in total surface area of tumors between the animals in the control groups (A and B in Fig. 1) were compared using the Kruskal-Wallis test followed by the Dunn test. The treatment effect is thus considered completely confounded with the differences between the groups of animals used within each experiment.
each modality of treatment and is therefore tested against the error term estimated from the animals within the groups. ANOVAs were performed on ranks due to the lack of normality of the residuals and/or heteroscedasticity of the distribution. Posteriori pairwise comparisons were also performed using least square means statistics.

Significance of the difference observed was accepted for $\alpha$ lower than 5%. All statistical tests were performed using the SAS software (SAS Institute, Cary, NC).

RESULTS

Experiment 1. Whereas estrone (group B) caused a 514% increase in ZR-75-1 tumor size ($P < 0.01$) during the 9-month treatment period (Fig. 4), daily oral administration of 300 $\mu$g of the antiestrogen EM-800 (group D) completely prevented tumor growth. In fact, not only was tumor growth completely prevented by EM-800, but a 73% reduction of average tumor size below the initial value was observed ($P < 0.0001$). This effect was not significantly different from the one achieved by ovariectomy alone (group A), which reached a value 83% below initial tumor size ($P < 0.0001$).

Radiation therapy (group C), during the first 56 days, markedly reduced average tumor size by 36% from the original value. This effect was followed by a plateau for about 100 days before a slight increase in average tumor size was seen, which reached 89% of the original value after 259 days of treatment. The average tumor size in animals who had radiotherapy alone (group C) was significantly lower than in the estrone-treated control group (group B, $P < 0.0001$) but significantly higher than in the OVX control group (group A, $P < 0.005$), the EM-800-treated group (group D, $P < 0.05$), and all combination groups (groups E, F, and G, $P < 0.005$).

When EM-800 at the daily dose of 300 $\mu$g was combined with radiation therapy at the start of treatment (group E), average tumor size was reduced by 98% after 9 months compared with initial size ($P < 0.0001$). Moreover, tumor size of this combination therapy group (group E) was significantly inferior by 23% (98% versus 75% below initial size) compared with that achieved in animals treated with EM-800 alone ($P < 0.005$). Although not reaching the level of statistical significance ($P = 0.057$), this 98% value was lower by 15% compared with the OVX control group (group A). The addition of EM-800 to mice initially treated with radiation therapy alone for 35 days (group F), or conversely, the addition of radiation therapy to mice treated with EM-800 alone for the same time period of 35 days (group G), caused a greater decrease in tumor size than either modality alone (groups C and D) but less than when both treatments were started simultaneously at start of therapy. In fact, starting EM-800 35 days after the start of radiation treatment caused a 95% decrease in tumor size (group F). On the other hand, when radiotherapy was started in mice already treated for 35 days with the antiestrogen, a 86% decrease in tumor size was observed [group G; $P < 0.005$ for both groups (groups F and G) versus radiation alone (group C)] but not statistically different from EM-800 alone (group D).

It is also of interest to analyze the categories of responses achieved under the experimental conditions described above. In the absence of estrone supplementation in OVX animals (group A), complete, partial, and stable responses were achieved in 50, 39, and 11% of tumors, respectively, and no tumor progressed (Fig. 5). In OVX animals supplemented with estrone (group B), 100% (17 of 17) of tumors progressed. In the EM-800-treated group (group D), on the other hand, complete, partial, and stable responses were seen in 40, 40, and 20% of tumors, respectively, and as seen in the control OVX group, no progressing tumor was seen. For the group treated with radiation therapy alone (group C), 20, 33, and 27% of tumors achieved complete, partial, and stable responses, respectively, whereas 20% of
tumors (3 of 15) progressed. When the combination of radiation therapy and antiestrogen was initiated on the first day of the experiment, 86% (12 of 14) of tumors disappeared, and only two tumors remained in the partial regression category (14%) after 259 days of treatment.

The two groups of animals who received the combination therapies after a pretreatment of 35 days (groups F and G) presented responses not statistically different from group D, who received EM-800 alone, but better than radiotherapy alone. In fact, complete, partial, and stable responses were observed in 33, 58, and 8% of tumors, respectively, in the group that received the antiestrogen 35 days after 10 Gy irradiation (group E), whereas the addition of radiotherapy to mice treated previously with EM-800 for 35 days (group G) resulted in a 44, 44, and 11% response in the complete, partial, and stable categories, respectively. It is interesting to note that in all groups treated with EM-800 as well as in the OVX control group, there were no progressing tumors.

To test the viability of the tumors that disappeared during treatment, each mouse bearing a tumor that disappeared during the course of the experiment was isolated 3 weeks after complete regression before receiving a s.c. E2 implant. A total of 44 mice were thus isolated during the course of the experiment. It is noteworthy that none of the tumors that showed a complete response during the course of the experiment in the OVX control group (group A, n = 9 of 18) as well as in the groups treated with radiotherapy alone (group C, n = 3 of 15), EM-800 after 35 days of radiotherapy (group F, n = 4 of 12), and the combined treatment initiated on the first day (group E, n = 12 of 14) reappeared at later time intervals (Fig. 6). Thus, it is quite remarkable that only 2 of 44 tumors reappeared after 40 and 25 days of E2 stimulation, respectively. It is important to note that 12 mice were isolated and stimulated with an E2 implant in the combined treatment group E, and no tumor appeared. Of these mice, none (6%) were observed up to 120 days and were thus considered cured, whereas only three mice did not survive 50 days (Fig. 6).

The dose of radiation therapy used had no apparent effect on the general health of mice, and no death was associated with the treatment. Histopathological examination of tissues directly in contact with radiation (skin) and potentially in contact (liver) showed no significant anomaly in any of the experimental groups. No significant effect of radiation therapy or EM-800 treatment was observed on body weight adjusted for tumor weight (data not shown). On the other hand, treatment with 300 mg of EM-800 daily alone or in combination resulted in a complete blockade of the stimulatory effect of estrone on uterine weight. In fact, there is no significant difference in uterine weight among all groups who received EM-800 during the course of the experiment (1.4 ± 0.1 to 1.8 ± 0.1 mg/g of body weight for groups D to G) and that observed in the OVX control group (group A) without estrone stimulation (1.7 ± 0.2 mg/g of body weight). On the other hand, uterine weight of mice treated with estrone increased to 12.1 ± 0.8 mg/g of body weight, and treatment with radiation therapy alone did not change this value significantly (10.6 ± 0.7 mg/g of body weight; Fig. 7).

**Experiment 2.** As illustrated in Fig. 8, during the 137-day estrone stimulation period (from day −137 to day 0), average tumor size of all
groups increased by 338% ($P < 0.001$) from 28.4 ± 1.1 mm$^2$ to 92.1 ± 6.2 mm$^2$. Then, one group of animals was used as control without estrone stimulation (control OVX), while the four other groups continued to be treated with s.c. estrone alone or in addition with daily oral doses of EM-800 and/or 10 Gy/35 days radiation therapy for 120 extra days as described in “Materials and Methods.” All data on tumor size are expressed as the percentage of the values recorded on day 0, when treatments were initiated.

Estrone supplementation for 120 days (OVX + E$_1$) led to a continuous progressive increase in average tumor size that reached 169% of the value measured on day 0. In comparison with the first experiment, the increase in tumor size in this group corresponded to 617% of the value measured on day −137. On the other hand, 120 days of withdrawal of the estrogenic stimulation (OVX control group) led to a progressive decrease in average ZR-75-1 tumor size by 51% ($P = 0.01$) of the pretreatment value. The 45% decrease of mean tumor area observed during treatment with a daily 300-μg dose of EM-800 in estrone-supplemented OVX mice ($P < 0.01$) is almost superimposable to the value observed in control OVX mice (51% decrease), thus indicating complete neutralization by EM-800 of the effect of estrone. On the other hand, treatment with the 10 Gy of radiation therapy/35 days caused a 17% decrease in tumor size (not significant). It is of interest to note that the inhibition pattern of tumor growth in the radiation therapy-treated group of the second experiment is similar to that observed in the first experiment (group C). In fact, an average tumor size at 68% of the initial value was measured after the first 60 days of radiation therapy, followed by a slow increase of tumor size to reach 83% at the end of the 120-day experiment. In the animals who received the daily dose of 300 μg of the antiestrogen in combination with 10 Gy of radiation therapy each 35 days, average tumor size was reduced by 62% compared with the size measured on day 0 ($P < 0.05$). The 24.9 ± 11.0 mm$^2$ value measured at the end of the experiment was similar to the original tumor size (28.4 ± 1.1 mm$^2$) observed before the initial estrogenic stimulation on day −137 (Fig. 8).

Concerning the categories of responses achieved in the second experiment, it is of interest to remember that the mice were treated with the above-indicated treatments for 120 days starting only after 137 days of estrogenic stimulation. Thus, possibly due to the larger size of the tumors, no tumor regressed completely in any group. Although the duration of treatment was much shorter, treatment with radiotherapy, EM-800, and the combination of both treatments de-
creased the number of progressing tumors from 50% in the control OVX animals supplemented with estrone to values of 20, 0, and 0%, respectively (data not shown). Stable responses, on the other hand, were measured at 25, 80, 37.5, and 33.3% in the control E1-supplemented mice and in the three groups of animals who received the above-indicated treatments, respectively. Partial responses, on the other hand, increased from 25% in the estrone-supplemented mice to 62.5 and 66.7% in the animals receiving EM-800 alone or in combination with radiation therapy, respectively, whereas no partial response was observed in the group of animals that received radiotherapy alone. In control OVX mice, the rates of partial and stable responses were measured at 62.5 and 37.5%, respectively, and no progression was seen (data not shown).

No significant effect of radiation therapy or EM-800 treatment was observed on body weight adjusted for tumor weight (data not shown). Daily administration of 300 μg of EM-800 alone or in combination with radiotherapy to estrone-supplemented OVX mice resulted in a complete blockade of the stimulatory effect of estrone on uterine weight (2.6 ± 0.2 mg/g of body weight and 2.2 ± 0.2 mg/g of body weight, respectively). This value is similar to that measured in OVX control mice without estrone supplementation (2.1 ± 0.2 mg/g of body weight). On the other hand, uterine weight in the estrone-treated control group and in the radiation therapy group increased from 2.1 ± 0.2 mg/g of body weight in OVX animals to 12.5 ± 1.6 mg/g of body weight and 9.9 ± 1.4 mg/g of body weight, respectively.

DISCUSSION

The present study clearly shows that although the pure antiestrogen EM-800 alone and radiation therapy alone have major beneficial effects on the growth of human breast cancer xenografts in nude mice, the combination of both therapeutic modalities leads to even greater benefits. In fact, it is quite remarkable that 12 of the 14 (86%) tumors disappeared, thus indicating a cure in the group of animals who received the combination therapy.

The potential of greater benefit of combined treatments that act via different mechanisms is supported by the evidence that breast cancers are composed of genetically and phenotypically heterogeneous subpopulations of tumor cells, especially when the tumors have spread outside the original site of cancer development. It thus seems reasonable to target, after surgery, the most estrogen-sensitive cell population with a pure antiestrogen while, at the same time, another treatment such as radiation therapy and/or chemotherapy can be added.

Treatment of breast cancer cells in vitro with EM-800 alone resulted in a slowing of the cell cycle and a consequent decrease of cell proliferation (20). This arrest in the G0-G1 phase of the cell cycle is caused by blockade of the estrogen receptor whereas radiation therapy induces damage in the G2 phase of the cell cycle (21). If, as frequently hypothesized, EM-800 would cause G0-G1 arrest and radiation therapy acts at the G2 phase of the cell cycle, the antiestrogen would prevent cells from progressing to the G2 phase, the cancer cells potentially becoming less sensitive to the effect of radiation therapy. Such a hypothesis, however, is not valid, as shown by the present in vivo data. In fact, the present data clearly show that treatment with EM-800 not only slows tumor growth but can cause complete disappearance of a large proportion of tumors or cure. Most importantly, proof of the complete disappearance of tumors is provided by the lack of reappearance of tumors under estrogen stimulation in 87% of cases.
(seven of eight tumors) in the group of mice treated with EM-800 alone.

In contrast with a study in a model of prostate cancer, where radiation therapy was given in combination with chemotherapy that resulted in cessation of cell proliferation without manifestation of cell death (22), our study with EM-800 alone decreased tumor volume through a cytotoxic effect in the same proportion as that observed in the OVX control group of animals. It is most interesting that the addition of radiation therapy to complete blockade of estrogenic action by EM-800 decreased tumor size and induced the disappearance of tumors to a greater degree than achieved by ovariectomy. The present data clearly show that blockade of estrogenic action by a potent and pure antiestrogen is not only cytostatic, as commonly thought, but is strongly cytotoxic and leads to disappearance of tumors. It is also important to indicate that an optimal effect has been observed when EM-800 and radiation therapy are administered simultaneously at the start of treatment.

The present data support the design of clinical adjuvant studies where radiation therapy will be combined to pure antiestrogen therapy after surgery in women with node-positive breast cancer or in women having moderate or high risk of remaining local disease after breast conservative surgery. Considering the excellent tolerance observed with EM-800 in women, the optimal regimen could well be the use of the antiestrogen both as neoadjuvant (before surgery) and adjuvant (after surgery) therapy, with radiation therapy being part of the adjuvant regimen.

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