

EFFECT OF TREATMENT SEQUENCE WITH RADIOTHERAPY AND THE ANTIESTROGEN EM 800 ON THE GROWTH OF ZR 75 1 HUMAN MAMMARY CARCINOMA IN NUDE MICE

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We demonstrated previously that continuous administration of EM-800, a SERM having pure antiestrogenic activity in the mammary gland and endometrium in combination with monthly radiotherapy caused a greater inhibition of human ZR 75 1 tumor growth in nude mice than either therapy used alone. To further optimize therapy, we have now examined the effect of various treatment sequences to determine the optimal treatment regimen in the same model. EM 800 was given at the maximally effective oral dose of 300 µg daily. External beam radiation therapy (RTX) was carried out (2 Gy/tumor/day, 5 days per week for 3 weeks) for a total of 30 Gy/tumor delivered directly to the tumor while shielding the rest of the animal body. There was no evidence of RTX-related morbidity. Continuous treatment with EM 800 was initiated either 3 weeks before or at the same time as RTX, immediately after RTX, or 3 weeks before and immediately after RTX. After 156 days of treatment, EM 800 alone caused a 75% decrease in average tumor area, an effect equivalent to that achieved by ovariectomy. RTX alone, on the other hand, caused a transient 30% decrease in tumor area regardless of treatment sequence, whereas combined treatment with EM 800 and RTX was superior to either treatment alone. Combined treatment with EM 800 and RTX both started on Day 1 caused the greatest (88%), most rapid (50% in 2 weeks) and sustained decrease in tumor size. The present data indicate that optimal reduction in breast tumor size is achieved by continuous administration of EM 800 and RTX started simultaneously on Day 1.

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Key words: breast cancer; antiestrogen; EM-800; SCH.57050; EM-652; SCH 57068; radiotherapy; xenografts; ZR-75-1; nude mice

Breast cancer is the most frequently diagnosed cancer in women in the United States, accounting for 30% of all cancers. It is estimated that 205,000 new cases of breast cancer will be diagnosed and 40,000 women will die from this disease in 2002.¹ Despite the availability of treatment modalities such as surgery, radiation therapy, chemotherapy and hormone therapy, the rate of disease recurrence with metastases after a few years from the first treatment remains high, possibly due in part to the heterogeneity of the cancer cell populations that respond differently to each therapy.² It is thus reasonable to believe that the combined use of more than one effective treatment modality that block cell proliferation and induce apoptosis via different mechanisms should lead to a greater, more rapid and more sustained inhibition of tumor growth.

Estrogens are well known to play the predominant role as stimulators of breast cancer development and growth.³ It is also well recognized that estrogen deprivation causes regression of a large proportion of breast tumors, especially at the localized stage. In the near future, it is likely that a less invasive and a more efficient method to achieve blockade of estrogen action, will be the use of pure antiestrogens.^{4–6} In this context, the third generation SERM (selective estrogen receptor modulator) EM-800, possesses the most potent known antiestrogenic activity both *in vitro* as well as *in vivo* in all the models used.^{4,7–12} Most interestingly, this orally active antiestrogen is the only non-steroidal antiestrogen that exerts pure antiestrogenic activity in both the human mammary gland and endometrium.^{4,13–15} EM 800 has also been shown to exert beneficial effects in women with breast cancer who had failed tamoxifen.⁴

Radiotherapy, on the other hand, is well known for its beneficial effect in breast cancer, especially as adjuvant to surgery. Traditionally, radiobiologists believed that radiotherapy is most effective against dividing cells. Therefore, because hormone blockade markedly reduces cell proliferation, one could assume that hormone-sensitive tumor cells lose their radiosensitivity under hormone deprivation. No clinical evidence, however, supports this hypothesis. On the contrary, in 3 prospective randomized trials of the American Radiation Therapy Oncology Group and of the European Organization for Research and Treatment of Cancer (EORTC), androgen deprivation combined with radiotherapy at start of treatment resulted in a marked increase of local tumor control and disease-free survival compared to irradiation alone in patients with locally advanced prostate cancer.^{16–20} Most importantly, in these 3 studies, a decreased incidence of death from prostate cancer was observed. In fact, the first study demonstrated to prolong life in prostate cancer is the one where long-term androgen blockade (3 years or more) was associated with RTX compared to RTX alone.^{16,19} In fact, in the EORTC study, death from prostate cancer at 5 years was reduced by as much as 77%. Moreover, in Bolla's and Pilepich's studies, androgen blockade was started at the same time as radiotherapy.^{16–20}

Moreover, that optimal control of breast cancer growth can potentially be achieved by the combined and simultaneous use of therapeutic agents that block cell proliferation and induce apoptosis via different mechanisms was demonstrated in other tumor models with the combination of chemotherapy and radiation therapy²¹ as well as following the association of tamoxifen and chemotherapy in human breast cancer.²²

We found previously that the pure SERM EM-800 alone, as well as monthly cycles of radiation therapy alone and cyclophosphamide treatment alone repeated every 2 weeks independently have major beneficial effects on the growth of ZR-75-1 human breast cancer xenografts in nude mice.^{8,23,24} The combination of any of the 2 cytotoxic modalities with the antiestrogen EM 800 led to a greater decrease in tumor size and induced the disappearance of tumors to a greater extent in nude mice.^{8,23} These previous data support the suggestion that breast cancer is composed of genetically or phenotypically heterogeneous subpopulations of tumor cells, which respond differently to each treatment modality. It should also be mentioned that an inverse relationship exists be-

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tween tumor volume and the success of tumor control by irradiation (or induced by EM-800), with smaller tumors requiring lower doses of radiation for their eradication.⁸ A similar finding has been found in mouse mammary carcinoma Shionogi tumors.²⁵

To obtain information on the important question of optimal timing of estrogen blockade and radiotherapy, our present study investigates the potential time-dependent interactions between the antiestrogen EM-800 and radiation therapy to determine the optimal timing of the combined treatment regimen. To achieve this goal, we have studied the effect of several sequences of treatment with EM 800 as neoadjuvant, concomitant, adjuvant and combined neoadjuvant-adjuvant to radiotherapy on the growth of the well-characterized estrogen-sensitive ZR-75-1 human breast xenografts in nude mice. In our study, we included 48 tumors on 24 mice per group to obtain a statistically highly powerful experimental design. EM 800 was given orally at the maximal effective dose of 300 μg daily. Continuous treatment with EM 800 was administered either 3 weeks before or at the start of radiotherapy, immediately after radiotherapy, or 3 weeks before and immediately after radiotherapy.

MATERIAL AND METHODS

Human breast cancer ZR-75-1 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 medium.²⁶ 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) FBS and incubated under a humidified atmosphere of 95% air/5% CO₂ at 37°C. Cells were passaged weekly and harvested at 85–90% confluence using 0.083% pancreatin/0.3 mM EDTA. 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 nu/nu Br athymic mice (28- to 42-day-old) were obtained from Charles River, Inc. (Saint-Constant, Québec, Canada). The mice (4–5 per cage) were housed in vinyl cages equipped with air filter lids, which were kept in laminar airflow hoods and maintained under pathogen-limiting conditions. The photoperiod was 12 hr of light and 12 hr of darkness (lights on at 07:15). Cages, bedding and food (Agway Pro-Lab R-M-H Diet 4018) were autoclaved before use. Water was autoclaved and provided *ad lib*. Bilateral ovariectomy was carried out under isoflurane-induced anesthesia. At the time of ovariectomy, an implant of estradiol (E₂) was inserted subcutaneously 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 × 10⁶ ZR 75 1 (passage 93) cells were inoculated subcutaneously in 0.1 ml of RPMI-1640 medium + 30% Matrigel on both flanks of each mouse through a 2.5-cm-long 22-gauge needle. After 4 weeks, the E₂ implants were replaced in all animals by estrone containing implants of the same size (E₁:chol, 1:25, w:w). Randomization and treatments were started 1 week later. These implants provide a physiological dose of estrogen that maintains uterine weight at the level found in intact animals.

Treatments

On the day before the experiment (Day 0), 192 mice bearing ZR-75-1 tumors of an average area of 20.27 ± 0.36 mm² (range 3.6–43.4 mm²) were assigned to 8 groups by randomization stratified with respect to tumor size, each containing 24 mice (46–48 tumors). Tumor measurements were carried out weekly using Vernier calipers. Two perpendicular tumor diameters were measured and tumor area (in mm²) was calculated using the formula L/2 × W/2 × π . Individual tumor areas calculated on Day

0 of the experiment were assigned a value of 100%. On Day 1 of the experiment, E₁ implants were removed from mice assigned to the OVX control group (Group 1). Ovariectomized animals supplemented with estrone were used as a model of postmenopausal women where estrone is the main circulating estrogen. Mice of Groups 1 and 2 (E₁ control) received oral vehicle once daily whereas mice of Group 3 received 300 μg EM-800 orally once daily for the entire study. The mice assigned to Groups 4–8 received radiotherapy (RTX), alone or in combination with EM-800, as indicated in Figure 1. The 300 μg dose of EM 800 is the one giving complete inhibition of estrone-induced stimulation of breast tumor growth.

Hormonal therapy

EM-800 ((+)-7-pivaloyloxy-3-(4'-pivaloyloxyphenyl)-4-methyl-2-(4''-(2''-piperidinoethoxy)phenyl)-2H-benzopyran) was synthesized in the medicinal chemistry division of the Oncology and Molecular Endocrinology Research Center, Laval University Hospital (CHUL), Québec, Canada. EM-800 was suspended in autoclaved 0.4% (w/v) methylcellulose vehicle at a concentration of 1.5 mg/ml. EM-800-dosed mice received 0.2 ml of the EM-800 dosing suspension once daily by oral gavage (12 mg/kg). All the other mice received 0.2 ml of the vehicle alone. This dose provides average serum levels of EM-652, the active antiestrogen, of 5 ng/ml (AUC_{0–24 hr} = 120 ng.hr/ml) whereas the 20 and 40 mg daily doses used in postmenopausal women⁴ provide average serum EM-652 levels of about 7.5 and 15 ng EM-652/ml, respectively.

Radiation therapy

The radiation therapy method used in this experiment was derived from a technique described previously.⁸ This new technique allows to administer external beam radiation to 24 mice simultaneously under the pathogen-free conditions required by nude mice. The mice were manipulated in a portable Biobubble equipped with HEPA filtration (BioBubble Inc., Fort Collins, CO). The mice were anesthetized by infusing isoflurane directly into the microisolators. The anesthetized mice were transferred to a specially-constructed Plexiglas irradiation box designed to hold 24 mice. The mice were then maintained under isoflurane-induced

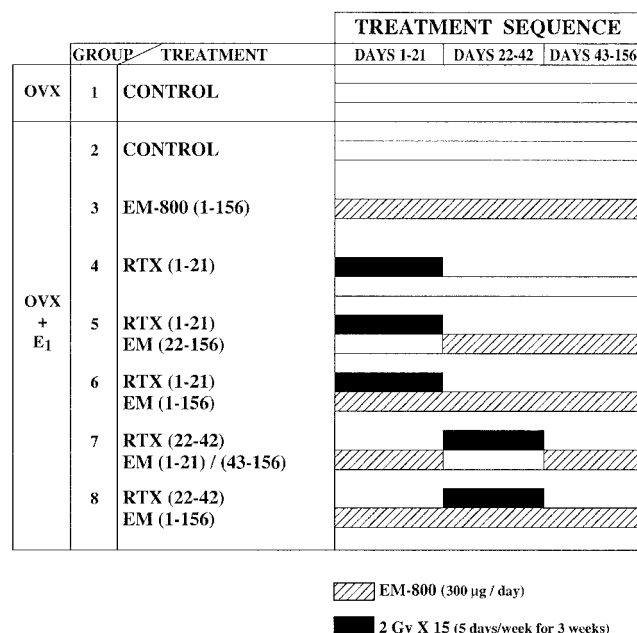


FIGURE 1 – Study design.

anesthesia throughout the radiation treatment. The mice were shielded from the radiation source by a perforated lead plate, which exposed only the mammary tumors. Radiotherapy was administered in 2 Gy fractions for 15 sessions (5 days per week for 3 weeks) of 383 MUs using the 6 MeV electron accelerator (DSP of 130 cm) of the radio-oncology service of the Hôtel-Dieu Hospital in Quebec City. To better appreciate the possible interaction between EM 800 and radiotherapy, the radiation treatment design was not established to obtain a maximal effect. These radiation parameters were established to administer a homogeneous radiation dose to each individual tumor. This treatment regimen is estimated to correspond to half the total dose of radiation administered to cancer patients.

Response criteria

The response criteria were adapted from Dauvois *et al.*²⁷ In brief, complete regression identifies those tumors that were undetectable at the end of the experiment; partial regression corresponds to the tumors that regressed $\geq 50\%$ of their original size; stable response refers to tumors that regressed $< 50\%$ or progressed $\leq 50\%$; and progression indicates those tumors that progressed $> 50\%$ compared to their original size. The 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. Only mice that survived until Day 78 of the study were inserted in this classification.

Necropsy and histopathology analysis

After 156 days of treatment, the animals were anesthetized with isoflurane and killed by cervical dislocation. To characterize the effect of estrogen and antiestrogen on estrogen responsive tissues, vagina and uteri, were immediately removed at necropsy, freed from connective and adipose tissue and weighed. To assess the effect of radiation on the tissues surrounding the tumors, hepatic sections were collected in the 2 lateral lobes. Additionally, 2 sections of the skin overlying the xenografts in the flank region were collected in all animals. These collected tissues were rapidly fixed in 10% buffered formalin and then processed in a tissue processor, embedded in paraffin, cut in 4 μm thick sections and stained with hematoxylin and eosin for histopathological examination.

Statistical analyses

The change in total tumors surface areas between Day 1 and Day 156 were analyzed according to an ANOVA for repeated measurements. The model included the treatment, time, and time-treatment interaction effects plus the term to account for the strata at randomization. The significance of the different treatments effects at 156 days was thus tested by the time-treatment interaction. Analysis of the residuals indicated that the measurements on the original scale were not fitted for analysis by an ANOVA nor any of the transformations that were tried. The ranks were therefore selected for the analyses. The effect of the treatments on the body, uterus and vagina weights was assessed by a 1-way ANOVA including also the strata at randomization. Uterus and vagina weight were expressed per 100 g of the body weights. A posteriori pairwise comparisons were carried out using least square means statistics. The overall Type 1 error rate (α) was controlled at 5% to declare significance of the differences. All calculations were carried out using Proc MIXED on the SAS Software (SAS Institute, Cary, NC).

RESULTS

Effect on tumor size

Effect of estrone supplementation. Estrone stimulation caused a 305% increase in ZR-75-1 tumor size ($p < 0.001$) during the 156-day treatment period (Fig. 2a).

Effect of radiation therapy. Radiation therapy alone in estrone-supplemented mice (Group 4) markedly reduced average tumor

size by 51% from the original value ($p < 0.001$) during the first 44 days although the last treatment was on Day 19. This transient inhibitory effect was followed by an increase in tumor size which, at the end of the experiment (Day 156) reached 149% of the original value ($p < 0.001$) (Fig. 2a,b). The average tumor size in animals that received radiotherapy alone (Group 4) was significantly lower than that in the estrone-supplemented control group ($p < 0.001$ vs. Group 2). With RTX alone (Group 4), tumor size was significantly higher than that of the OVX control group (Group 1) ($p < 0.001$, OVX vs. OVX + E₁ + RTX).

Effect of hormonal therapy. Daily oral administration of 300 μg of the antiestrogen EM-800 (Group 3) completely prevented tumor growth. In fact, not only tumor growth was completely prevented by EM 800, but a 75% reduction in average tumor size below the initial value was observed ($p < 0.001$). This effect was superimposable to the one achieved by ovariectomy alone (Group 1), which reached a value of 76% below initial tumor size at Day 156 ($p < 0.001$) (Fig. 2a,c).

Effect of combined treatment initiated with EM-800 or radiotherapy alone. The addition of EM 800 to mice initially treated with radiation therapy alone for 21 days (Group 5) (Fig. 2b) or conversely, the addition of radiation therapy at 22 days to mice initially treated with EM 800 alone for the same time period (Groups 7,8) (Fig. 2c), caused a greater decrease in tumor size at the end of the experiment (84%, 89% and 88%, respectively) than either modality alone (Group 3, $p < 0.03$ and Group 4, $p < 0.001$).

It can be noticed in Figure 2b,c that a significant decrease was observed only 3 weeks after the addition of the second therapy (EM 800 in Group 5 and RTX in Groups 7 and 8), compared to each treatment alone. In fact, the addition of EM 800 to radiation therapy (Group 5) or the addition of radiotherapy to continuous hormonotherapy (Group 8) caused an additional 13% decrease in mean tumor size compared to each therapy alone on Day 44 (36% (Group 5) vs. 49% (Group 4) and 33% (Group 8) vs. 46% (Group 3), $p < 0.03$).

When EM-800 was interrupted after 21 days, before the initiation of radiation therapy for 3 weeks (Group 7), no difference was observed in the decrease of tumor size during the 21 days of radiation therapy in comparison with continuous EM 800 administration (Group 3) (44% vs. 46% on Day 44) during this period. Despite the fact that both groups reached the same decrease after 44 days of treatment and they received thereafter the same treatment for the rest of the study, it is noteworthy that at the end of the experiment (Day 156), the addition of RTX in the combination Group 7 led to 14% lower values than in the EM 800 alone-treated group (Group 3) (89% vs. 75% inhibition $p < 0.009$) (Fig. 2c). The inhibition achieved was smaller, however, than when both EM-800 and RTX were started on Day 1 (Fig. 2c).

Effect of combined treatment initiated with both therapies. When EM-800 at the daily dose of 300 μg was combined with radiation therapy at start of treatment (Group 6), average tumor size was reduced by 95% after 156 days compared to initial size ($p < 0.001$). Moreover, the tumor size of this combination therapy group (Group 6) was significantly lower by 20% and 19% compared to the values achieved in animals treated with EM 800 alone and the OVX control group, respectively ($p \leq 0.004$) (Fig. 2a).

Although 21 days of treatment with EM 800 alone and radiotherapy alone (Groups 3 and 4) decreased mean tumor size by 41% and 45%, respectively ($p < 0.001$), the combined therapy initiated at Day 1 decreased tumor size by 57% ($p < 0.001$) vs. initial size ($p < 0.005$ vs. both groups receiving monotherapy) (Fig. 2a).

Effect on categories of response

It is also of interest to analyze the categories of responses achieved under the experimental conditions described above. In OVX animals supplemented with estrone (Group 2), 74% of tumors (28 of 38) progressed, and only 16%, 8% and 2% of tumors achieved stable, partial and complete responses, respectively.

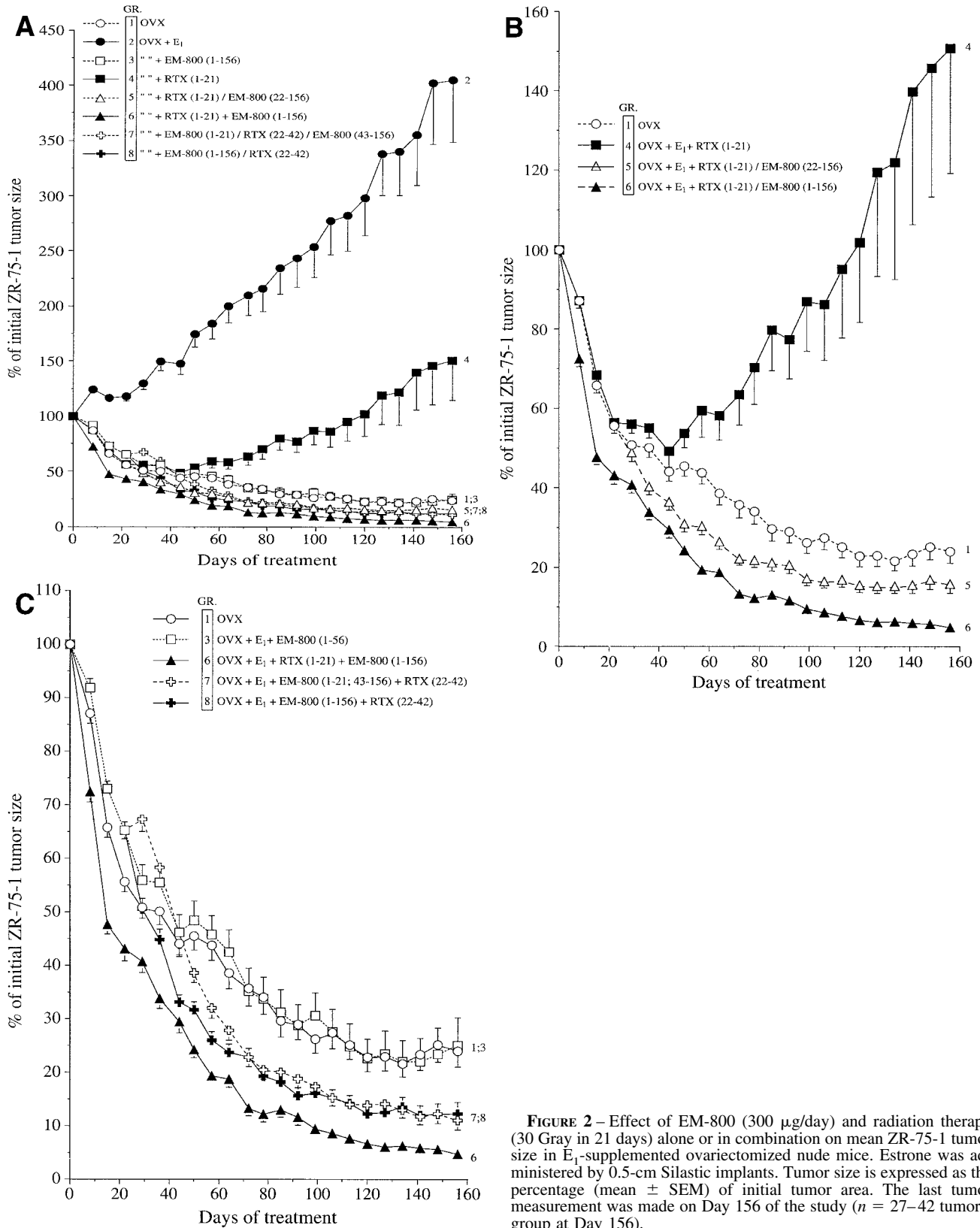


FIGURE 2—Effect of EM-800 (300 $\mu\text{g}/\text{day}$) and radiation therapy (30 Gray in 21 days) alone or in combination on mean ZR-75-1 tumor size in E₁-supplemented ovariectomized nude mice. Estrone was administered by 0.5-cm Silastic implants. Tumor size is expressed as the percentage (mean \pm SEM) of initial tumor area. The last tumor measurement was made on Day 156 of the study ($n = 27\text{--}42$ tumors/group at Day 156).

For the group treated with radiation therapy alone (Group 4), 12%, 14% and 49% of tumors achieved complete, partial and stable responses, respectively, whereas 25% of tumors (11 of 43) progressed.

In the absence of estrone supplementation in OVX animals (Group 1), complete, partial, and stable responses were achieved in 11%, 81% and 8% of tumors, respectively, and no tumor pro-

gressed (Fig. 3). In the EM 800 alone-treated group (Group 3), complete, partial, and stable responses were obtained in 39%, 46% and 15% of tumors, respectively, and as seen in the control OVX group, no progressing tumor occurred.

The 3 groups who received the combination therapies after monotherapy for 21 days (Groups 5, 7 and 8) presented complete regressions at levels of 22%, 34% and 39%, respectively. In the 3 groups, partial regressions were observed in 76%, 66% and 61% of tumors, respectively. It is most interesting to note that in all groups treated with EM-800 as well as in the ovariectomized control group that correspond to a total of 240 tumors, no single progression occurred. Moreover, in the 4 groups of combined therapy (Groups 5–8) 99.4% of tumors regressed (163 of 164) and only 1 remained stable, at 58% of its initial size (Group 5).

When the combination of radiation therapy and antiestrogen was initiated on the first day of the experiment (Group 6), 62% (24 of 39) of tumors disappeared, and 15 tumors remained in the partial regression category (38%) after 156 days of treatment, no stable or progressing tumor was seen.

Effect on body, uterine and vaginal weight

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 in comparison to the control group supplemented with estrone (Group 2).

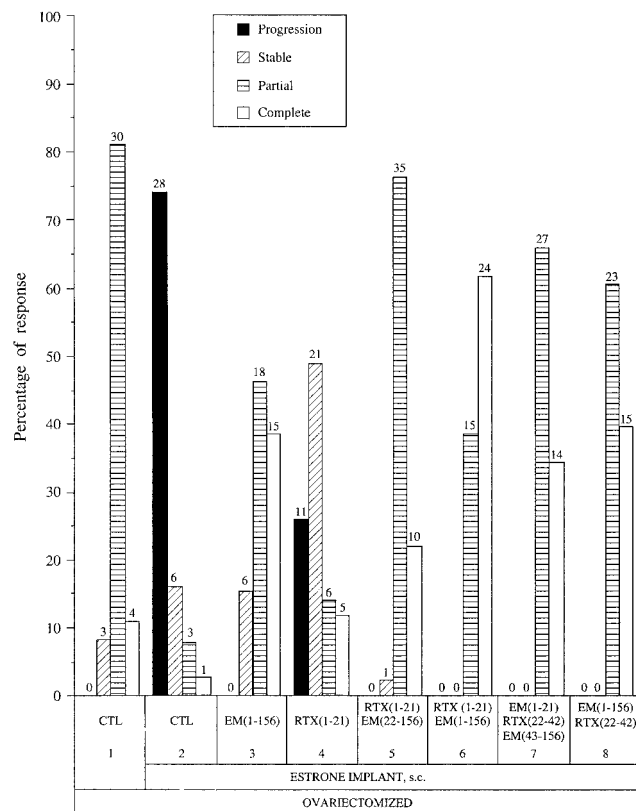


FIGURE 3—Effect of EM-800 (300 µg/day) and radiation therapy (30 Gray in 21 days), alone or in combination, on the category of response of human ZR-75-1 breast carcinoma xenografts in estrone-supplemented ovariectomized nude mice. Only mice that survived until Day 78 of the study were inserted in this classification whereas those that did not survive during this period were rejected.

Treatment with 300 µg of EM-800 daily alone or in combination resulted in a nearly complete blockade of the stimulatory effect of estrone on uterine weight (86% decrease in mean uterine weight vs. 90% in the OVX control group). In fact, there is a small but statistically significant difference ranging from 2–4% ($p \leq 0.004$) in uterine weight between all groups who received EM-800 and estrone, during the course of the experiment (1.4 ± 0.1 to 1.7 ± 0.2 mg/g of B.W. for Groups 3,5–8) and that observed in the OVX control group (Group 1) without estrone stimulation (1.2 ± 0.1 mg/g of B.W.). The mean value of uterine weight regression in all groups treated with EM-800 was 87%. Uterine weight of mice treated with estrone increased to 11.98 ± 0.59 mg/g of B.W. and treatment with radiation therapy alone did not change significantly this value (10.58 ± 0.57 mg/g of B.W.) (Table I). It is of interest to note that vaginal weight in the EM 800 alone-treated group (Group 3), and all EM 800 combination groups (Groups 5–8) was the same as in the ovariectomized control group (Group 1). Similar blockade of the stimulatory effect of estrone on vaginal weight was observed: 0.58 ± 0.03 to 0.69 ± 0.03 mg/g of B.W. for the EM 800 alone group (Group 3) or EM-800 in combination (Groups 5–8) (72–76% of vaginal weight regression) in comparison to a value of 0.69 ± 0.06 mg/g of B.W. (72% of regression) for the OVX control group (Group 1 without estrone stimulation). Vaginal weight of mice treated with estrone increased to 2.48 ± 0.2 mg/g of B.W. and treatment with radiation therapy alone did not change significantly this value (2.30 ± 0.13 mg/g of B.W.) (Table I).

DISCUSSION

The present study clearly demonstrates, in the human breast ZR-75-1 xenograft model, the net benefit of using the antiestrogen EM-800 in combination with radiation therapy at start of treatment compared to other sequences of treatment. Much evidence shows that combination of hormonal and radiation therapies is beneficial for prostate cancer treatment.^{16,19,20} In the European Organization for Research and Treatment of Cancer (EORTC) trial, carried out in patients with prostate cancer at Stage T3, overall survival at 5 years was increased from 62% in the group of patients who received radiation therapy alone to 79% in those who received androgen blockade using an LHRH agonist for 3 years and an antiandrogen for 1 month in association with radiotherapy at start of treatment.¹⁶ Similar observations have been made in 2 clinical trials from the Radiation Therapy Oncology Group (RTOG) in the subgroup of high Gleason score patients^{19,20} where overall survival was improved in the group of patients who received androgen blockade. Many studies have shown that time to progression is prolonged by early hormonal treatment of the disease.^{28–32}

Studies have shown that optimum treatment of high-risk breast cancer requires both locoregional and systemic tumor control.^{33,34} Estrogen blockade, using tamoxifen, is thus used currently in combination with postsurgical radiation to control potential micro-metastases, as a systemic approach complementary to local radiation. In fact, among ER positive patients, there was a significant reduction in treatment failures in the tamoxifen group compared to the groups allocated to radiation therapy or chemotherapy alone.³⁵

Tamoxifen appears to exert its cytostatic activity through competitive inhibition at the estrogen receptor level, thus resulting in segregation of cells in the G0/G1 phase of the cell cycle.^{36–39} It is now well accepted that interaction of tamoxifen with radiation therapy is more complex than suggested by simple single fraction clonogenic survival curves, some cooperative effect with radiation therapy being suggested by a decrease in Bcl-2 levels and in TGF-β secretion,^{40–42} two effects that could increase radiosensitivity of breast tissue.

Although tamoxifen has clearly shown clinical benefits in advanced breast cancer, its efficacy remains limited by the resistance to treatment that develops eventually in most women. The mixed estrogenic and antiestrogenic activities of this compound is

TABLE 1—EFFECT OF TREATMENT WITH EM-800 (300 µg, p.o., I.D.) AND RADIATION THERAPY (30 GY IN 21 DAYS), ALONE OR IN COMBINATION, ON BODY, UTERINE AND VAGINAL WEIGHT IN ESTRONE-SUPPLEMENTED OVARIECTOMIZED NUDE MICE

Treatment groups ²	n	Body (g)	Uterus		Vagina	
			(mg/g of BW)	Regression (%)	(mg/g of BW)	Regression (%)
OVX						
Control	18	29.3 ± 0.7	1.2 ± 0.1	90	0.7 ± 0.1	72
OVX + E ₁						
Control	14	27.4 ± 0.8	12.0 ³ ± 0.6	0.0	2.5 ³ ± 0.2	0
EM (1–156)	16	28.6 ± 0.9	1.7 ³ ± 0.2	85.8	0.7 ± 0.0	72
RTX (1–21)	18	27.0 ³ ± 0.5	10.6 ³ ± 0.6	11.7	2.3 ³ ± 0.1	8
RTX (1–21)/EM (22–156)	21	25.8 ³ ± 0.5	1.6 ³ ± 0.1	86.7	0.7 ± 0.0	72
RTX (1–21) + EM (1–156)	19	26.2 ³ ± 0.4	1.6 ³ ± 0.1	86.7	0.6 ± 0.0	76
EM (1–21)/RTX (22–42)	19	26.2 ³ ± 0.6	1.4 ³ ± 0.1	88.3	0.6 ± 0.0	76
EM (43–156)						
EM (1–156)/RTX (22–42)	19	27.3 ⁴ ± 0.7	1.6 ³ ± 0.1	86.7	0.7 ± 0.0	72

¹The data are expressed as means ± SEM (n = 14–21 tissues/group)—²OVX, ovariectomized; RTX, radiation therapy; EM, EM-800; BW, body weight.—³Experimental vs. OVX control mice, p ≤ 0.01.—⁴Experimental vs. OVX control mice, p ≤ 0.01.

thought to be, at least in part, responsible for this resistance.^{43–45} The mixed agonistic/antagonistic activities of tamoxifen are species-, tissue-, cell- and even gene-specific.^{46,47} In support of the clinical evidence for the estrogenic activity of tamoxifen in human breast cancer growth,^{48,49} tamoxifen and its active metabolite 4-OH-tamoxifen have been found to stimulate the growth of human breast cancer cells *in vitro* and *in vivo*.^{47,50–55} Tamoxifen may act as an estrogen agonist more frequently than generally thought and this may explain some of the apparent paradoxes of endocrine treatment such as response to second endocrine therapy and positive response to tamoxifen withdrawal.^{48,56} Despite these limitations, the antiestrogen tamoxifen has so far been the almost exclusive antiestrogen available in clinical practice. The benefits of tamoxifen after surgical treatment^{57–59} or postsurgical radiation therapy^{34,60} is well established. The positive responses observed with the pure steroidal antiestrogen Faslodex in patients with breast cancer who had failed with Tamoxifen indicate the advantages of using a pure antiestrogen for the treatment of this disease. Because EM-800 shows estrogen-like effects in the bones,⁴ it is reasonable to believe that this compound could show an advantageous profile compared to Faslodex for the treatment of breast cancer, especially in localized disease where long-term treatment is required.

A new generation of antiestrogens well known to have pure antiestrogenic activity in the mammary gland and in the endometrium and to have a beneficial estrogen-like effect on bone density and cholesterol, offer new possibilities over tamoxifen, especially for long-term administration of the compound for chemoprevention.⁴ Due to its pure antiestrogenic activity and its particularly high potency, it is reasonable to expect that EM-800 should not only be more efficient than tamoxifen to treat breast cancer but its use should also decrease the tamoxifen related risk of uterine carcinoma during long-term use in women.^{61,62} EM-800 is the only nonsteroidal antiestrogen showing no estrogenic activity in human Ishikawa endometrial carcinoma cells as assessed by changes in alkaline phosphatase activity, a well known estrogen-sensitive parameter.^{4,11} Moreover in long-term studies in the rat, mouse, and monkey, EM-800 shows a potent inhibitory effect on the uterus without any estrogenic activity.^{9,13,14,63,64}

Although combined *in vivo* treatments led to a greater decrease in tumor size than either treatment used alone,^{23,65,66} it has been observed previously that treatment of breast cancer cells *in vitro* with EM-800 alone, or other pure antiestrogens, resulted in a slowing of the cell cycle in the G₀/G₁ phase and a consequent decrease of cell proliferation.¹² This arrest of the cell cycle by antiestrogens would prevent cancer cells from progressing to the G₂ phase and thus potentially becoming less sensitive to the effect of radiation therapy that is thought to induce damage in the G₂ phase of the cell cycle. This hypothetical interaction between hormonotherapy and radiotherapy did not appear to be correct as

shown by our previous *in vivo* data.^{8,23} The present data clearly show that estrogen blockade does not interfere with the effects of radiation therapy.

It is important to remember that previous studies have shown that the ZR-75-1 breast tumors, which showed complete responses to EM-800, did not progress at later time intervals^{7,55} after the arrest of antiestrogen treatment. Most importantly, we have found recently that tumors that responded completely to EM-800 did not reappear after interruption of EM 800 treatment and challenge with estradiol;⁸ thus suggesting complete apoptosis or cure of the tumors by antiestrogen therapy.

In line with our objective of improved therapy of breast cancer with the new pure antiestrogens, we have investigated various aspects of combined therapy using the antiestrogen EM-800 and radiotherapy or chemotherapy. We have chosen the ZR-75-1 xenograft model as a well-characterized model of human breast cancer. As mentioned above, we have demonstrated in 2 previous studies that combination of continuous EM-800 with continuous radiation therapy or continuous chemotherapy (cyclophosphamide) was more effective than either treatment used alone and even superior to the ovariectomized control group.^{8,23} These recent studies differ from those reported previously, by the net advantage of using a highly potent and pure antiestrogen, namely EM-800 instead of a mixed antiestrogen-estrogen. In one of these studies, the combined radiation/antiestrogen treatment led to a 98% inhibition in tumor size with 86% (12 of 14) of tumors having disappeared.⁸ Those studies have shown cure of the cancer and not only arrest of cancer cell proliferation. The 9-month duration of repeated radiation therapy, however, was not representative of a clinical radiotherapy regimen. It was thus important to further investigate the same combination therapy under a radiation therapy regimen closer to the clinical situation by testing combination sequences and increasing the number of tumors per group (24–48) to improve statistical power. In our present study, radiation therapy was thus administered according to a protocol closer to the clinical setting but only for half of the normal period of radiation (30 Gy in 3 weeks) used for complete treatment in women.

We have clearly found that, in this model, combination of the pure antiestrogen and radiotherapy shows additional benefits. Moreover, the results of the 4 combination groups show a near complete control of tumor growth, with 99.4% of tumors having regressed and only 1 tumor remaining in the stable category although it had regressed to 58% of its original size. The efficacy of each treatment is well demonstrated in comparison with the estrone-supplemented control group and the ovariectomized control group. The results obtained with EM-800 alone show tumor size reduction superimposable to the OVX control group and no tumor progression. These data demonstrate the pure antagonistic effect of EM-800 in human breast cancer tissue because ovariec-

tomized mice correspond to the situation of complete absence of estrogen.⁶⁷

It was expected that the amplitude of the contribution of radiation therapy on tumor control could not be as large when used in combination with a pure antiestrogen in the mammary gland and endometrium such as EM-800 compared to a partial blocker of the class of tamoxifen.^{33,34,68} Despite the complete inhibition of estrogen action on tumor growth by EM 800, all combinations gave statistically better results than each therapy used alone. Such results suggest that there could be a greater heterogeneity of responsiveness to each therapy than expected. We possibly underestimate the importance of the subpopulation of cells that respond less efficiently to each therapy.

The present data show that the combination of EM-800 and radiotherapy both initiated at start of the experiment is more efficient than any other sequence combination tested. It is also interesting to note that the difference between each sequence of therapy was observed during the first 21 days, but all curves became similar at later time intervals. We conclude by these observations that starting treatment earlier in the course of the disease is the most efficacious strategy of treatment. Moreover, with EM-800 or radiation therapy alone, the mean tumor size decreases markedly during the first 40 days. Despite this marked early inhibition, the combination of both treatments administered together during the same period is significantly more efficacious during long term observation.

The comparisons made between sequential combinations using radiation therapy or EM-800 as first treatment provide new information about treatment sequence. In fact, despite a similar early inhibition by the 2 treatments used alone, thus leading to a equivalent mean tumor volume at 21 days, the combination group started with radiation therapy alone was slightly less efficacious at

the end of the 156 days of treatment than the 2 combination groups initiated with EM-800 alone (Group 5 vs. Group 7 and 8). Similarly, the efficacy of treatment combination initiated by radiotherapy alone was significantly lower than the combination of EM-800 and radiation therapy started on the first day (Group 5 vs. Group 6).

Five groups of responses can be summarized from our study: 1) the OVX estrone-supplemented group, where 75% of tumors were in progression; 2) the group receiving radiotherapy alone where 25% of tumors were in progression; 3) the groups where the estrogen-stimulation was completely abolished (OVX alone or the administration of EM-800 to estrone-treated OVX mice) in which no progressing tumors and a stabilization of the mean tumor size was seen; 4) the combination groups started with only 1 therapy, where mean tumor size decreased from 86% to 89% and where 20–40% of tumors completely regressed; and 5) the combination group initiated with both therapies in which more than 60% of the tumors disappeared and where the average tumor size was reduced by 95%.

In conclusion, a highly potent and pure antagonist of estrogen action in the mammary gland and endometrium such as EM 800 always shows important benefits when used in combination with radiation therapy, either as neoadjuvant, as concomitant or as adjuvant treatment. Moreover, despite the observation that all combinations were more efficient than each treatment used alone, our study clearly demonstrates that the best chance of cure in patients with breast cancer is likely to be achieved using a combination of a pure antiestrogen with radiation therapy started simultaneously as soon as possible, at start of treatment.

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