

LONG-TERM INHIBITORY EFFECT OF THE ORALLY ACTIVE AND PURE ANTIESTROGEN EM-800 ON THE GROWTH OF HUMAN BREAST CANCER XENOGRAPTS IN NUDE MICE

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The antiproliferative effect of the new antiestrogen EM-800 has been studied during 40 weeks of treatment on human breast carcinoma ZR-75-1 xenografts in ovariectomized nude mice supplemented with estrone (0.5 µg, s.c. daily). At the daily 50 µg (approximately 2.5 mg/kg) oral dose, EM-800 caused a complete inhibition of the 680% stimulatory effect of estrone on the growth of the ZR-75-1 human breast cancer xenografts. Complete response, defined as the complete disappearance of the tumors, was observed in 41% of tumors following treatment with the 50 µg dose of the antiestrogen, while a value of 26% was found in ovariectomized animals. The proportion of tumors showing progression at the end of 40 weeks of treatment decreased from 94% in the estrone-supplemented animals to 62%, 61% and 19% in the animals receiving the 5 µg, 20 µg and 50 µg daily doses of the antiestrogen, respectively. None of the tumors that showed a complete or a partial response progressed at later time intervals. The 50 µg daily dose of EM-800 nearly completely (93%) or completely (28% below the value in ovariectomized animals) reversed the stimulatory effect of estrone on uterine and vaginal weight, respectively. The disappearance of 41% of tumors in the group of animals that received the 50 µg daily dose of EM-800 indicates that the antiestrogen induces cell death or apoptosis in ZR-75-1 human breast cancer cells and that its action is cytotoxic and not only cytostatic. *Int. J. Cancer* 83:424–429, 2000.

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Breast cancer is the most frequent cancer in women, the odds of developing this cancer being 1 out of 9 women during their lifetimes. In fact, it is predicted that 176,300 new cases of breast cancer will be diagnosed in the USA in 1999, while 43,700 deaths are expected from this disease during the same period (Landis *et al.*, 1999). Breast cancer has thus become a major medico-social and public health problem. However, the present therapies of advanced breast cancer achieve significant positive clinical results in only 30–40% of cases, and these positive responses are usually limited to 12–18 months in patients with advanced disease.

Among all factors, estrogens are well recognized to play a predominant role in breast cancer development and growth (McGuire *et al.*, 1975). The existing surgical or medical ablative procedures do not permit complete elimination of estrogens in women. This limitation is due to the conversion of dehydroepiandrosterone (DHEA) and DHEA-sulfate of adrenal origin as well as ovarian androstenedione into estrogens in peripheral target intracrine tissues (Labrie, 1991). Considerable attention has thus focused on the mechanisms of action of estrogens and especially on the development of blockers of estrogen biosynthesis and action (Dauvois *et al.*, 1991; Labrie *et al.*, 1992; Wakeling and Bowler, 1988).

Since the first step in the action of estrogens in target tissues is binding to the estrogen receptor, 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, the antiestrogen by far the most widely used for the treatment of women with breast cancer, has clearly shown benefits in breast cancer therapy, its efficacy being comparable to that achieved with ablative and additive hormonal therapies (Furr and Jordan, 1984). Because of its low-side-effect

profile and a clinical efficacy comparable to other endocrine therapies, including oophorectomy and androgens, tamoxifen has become the endocrine treatment of choice for all stages of breast cancer (Furr and Jordan, 1984). Tamoxifen, however, is known to possess mixed estrogenic and antiestrogenic activities (Furr and Jordan, 1984; Labrie *et al.*, 1992), which are highly species-, tissue-, cell- and even gene-specific (Gottardis *et al.*, 1988). Moreover, while benefits of tamoxifen are observed on breast cancer in up to 40% of patients, the use of this compound has been associated with an increased incidence of endometrial carcinoma in women (Fornander *et al.*, 1993), an effect explained by the intrinsic estrogenic activity of the compound.

As a response to the need for pure antiestrogens, we have synthesized EM-800, a highly potent and specific antiestrogen (Gauthier *et al.*, 1997; Simard *et al.*, 1997a, b). This compound behaves as a highly potent and pure antiestrogen in human breast and uterine cancer cells. Indeed, EM-652, the active metabolite of EM-800, is the compound having the highest known affinity for the human estrogen receptor (Gauthier *et al.*, 1997) and a high potency to inhibit estrogen-stimulated proliferation of human ZR-75-1, MCF-7 and T-47-D breast cancer cells *in vitro* (Simard *et al.*, 1997a).

The present study examines the efficacy of increasing doses of EM-800 on the growth of xenografts of the human breast cancer cell line ZR-75-1 in nude mice. Daily oral administration of approximately 2.5 mg/kg of the compound caused a disappearance of 41% of the tumors, whereas progression was seen in only 19% of tumors at 40 weeks.

MATERIAL AND METHODS

Human breast cancer ZR-75-1 cells

ZR-75-1 human breast cancer cells obtained from the ATCC (Rockville, MD) were routinely cultured in phenol red-free RPMI-1640 medium. The cells were supplemented with 2mM L-glutamine, 1mM sodium pyruvate, 100 IU penicillin/ml, 100 µg streptomycin/ml and 10% (vol/vol) fetal bovine serum and incubated under a humidified atmosphere of 95% air/5% CO₂ at 37°C. Cells were passaged weekly by treatment with 0.05% 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 old) were obtained from Harlan Sprague Dawley (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 anesthesia achieved by i.p. injection of 0.25 ml/mouse of

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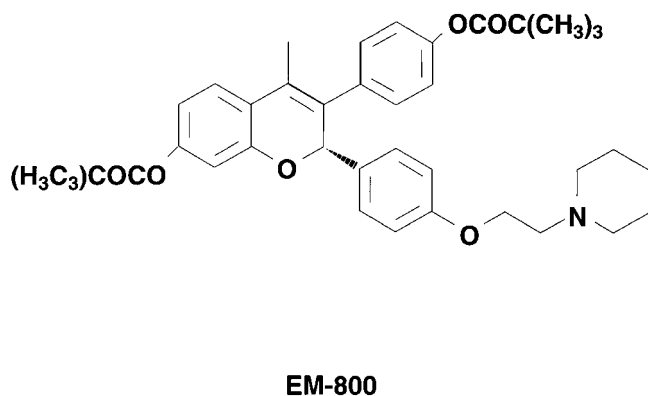


FIGURE 1 – Chemical structure of EM-800.

Avertin (amylic alcohol: 0.8 g/100 ml 0.9% NaCl; tribromo ethanol: 2 g/100 ml 0.9% NaCl).

ZR-75-1 cells (1.5×10^6) 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 culture medium containing 25% of Matrigel in the left flank of ovariectomized (OVX) animals through a 2.5-cm-long 20-gauge needle. To facilitate growth of the tumors, each animal received 10 μ g/day of estradiol (E_2) by s.c. injection in 0.9% NaCl-5% ethanol-1% gelatin for 5 weeks. Treatment with E_2 was interrupted for 10 days before the start of treatment with the indicated compounds. Mice bearing tumors of a diameter ranging from 0.2–0.7 cm were randomly assigned between 5 groups, each containing 15 or 16 mice.

Treatment

EM-800 [(+)-7-pivaloyloxy-3-(4'-pivaloyloxyphenyl)-4-methyl-2-(4'-(2''-piperidinoethoxy)phenyl)-2H-benzopyran] was synthesized in the medicinal chemistry division of our laboratory as described (Gauthier *et al.*, 1997). The compound analyzed under GLP conditions was > 99% pure. The structure of this antiestrogen is illustrated in Fig. 1. The compound was dissolved in 4% (v/v) ethanol-4% (v/v) polyethylene glycol 600-1% (w/v) gelatin-0.9% (w/v) NaCl. Animals of the OVX group received the vehicle (0.2 ml 5% ethanol-1% gelatin-0.9% NaCl) alone, while the animals of the 4 other groups received daily s.c. injections of 0.5 μ g of estrone in 0.2 ml of the same vehicle alone or in combination with daily oral doses of 5.0 μ g, 20 μ g or 50 μ g of EM-800. Ovariectomized animals supplemented with estrone were used as a model of post-menopausal women where estrone is the main circulating estrogen. The dose of estrone is the one giving an optimal effect on tumor growth, while the 50 μ g dose of EM-800 is the one giving near-complete inhibition of estrone-induced stimulation. Tumors were measured once a week with Vernier calipers. Two perpendicular diameters were recorded and tumor area (cm^2) was calculated using the formula $L/2 \times W/2 \times \pi$ (Dauvois *et al.*, 1991). 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. The size of tumors at the start of treatment was $0.14 \pm 0.01 \text{ cm}^2$. After 279 days of treatment, the animals were killed by decapitation. Uteri and vagina were then immediately removed, freed from connective and adipose tissue and weighed.

Response criteria

The criteria of response were as described in Dauvois *et al.* (1991): "complete" response corresponds to the category of tumors that became undetectable, "partial" response corresponds to tumors that regressed by 50% or more compared with their original size, while "stable" response is the category of tumors that have regressed by less than 50% and that have not progressed by

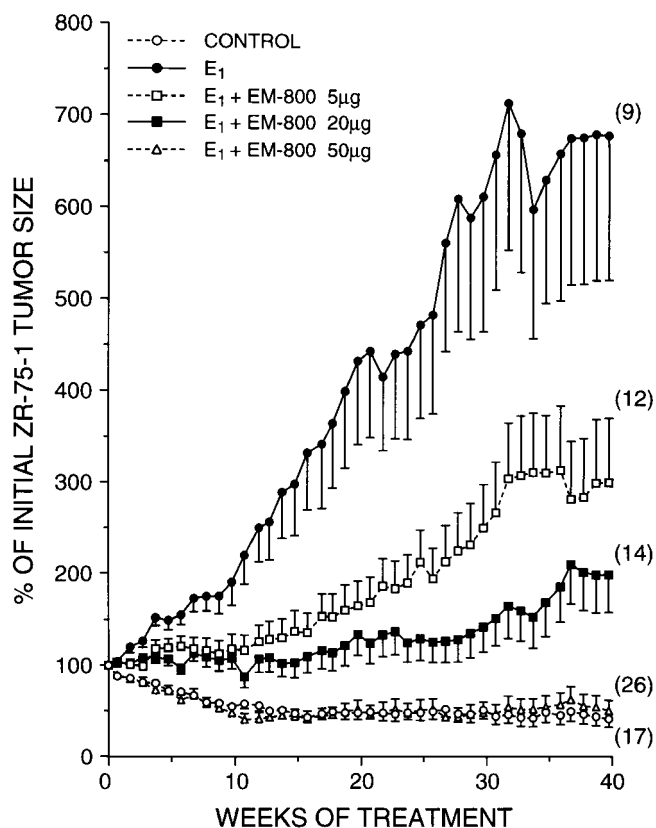


FIGURE 2 – Time course of the effect of treatment with the pure antiestrogen EM-800 at the daily oral dose of 5 μ g, 20 μ g, or 50 μ g for 40 weeks on the average size of ZR-75-1 human breast tumors in ovariectomized nude mice supplemented with a daily 0.5 μ g s.c. dose of estrone (E_1). Ovariectomized mice receiving the vehicle alone were used as additional controls. Results are expressed as percentage of pretreatment values (mean \pm SEM). The control, E_1 and 5 μ g, 20 μ g and 50 μ g EM-800 dose groups contained 23, 17, 21, 18 and 27 tumors at the start of treatment, respectively. Tumors that regressed completely were assigned a value of 0%. Each data point represents tumors measured in live animals. The number of tumors evaluated at week 40 is shown in parentheses.

25% or more. "Progression" is the category of tumors that progressed by more than 25%.

Statistical analyses

Statistical significance was calculated according to the multiple range test of Duncan-Kramer. All data are presented as means \pm S.E.M.

RESULTS

On day 0, ovariectomized mice bearing ZR-75-1 tumors were divided into 5 groups. The animals of 1 group received the vehicle alone, while the animals of the 4 other groups received s.c. estrone alone or in combination with daily oral doses of EM-800 ranging from 5–50 μ g. As illustrated in Fig. 2, estrogen supplementation with a daily s.c. dose of 0.5 μ g of estrone led to a progressive increase in average tumor size that reached 680% of the initial value at 40 weeks. On the other hand, withdrawal of estrogenic stimulation (OVX control group) led to a progressive decrease in average ZR-75-1 tumor size to 43% of the initial value at 16 weeks and 42% of pretreatment size at 40 weeks, the longest time interval studied. The values observed with the daily 50 μ g dose of EM-800 in estrone-supplemented OVX mice were almost superimposable

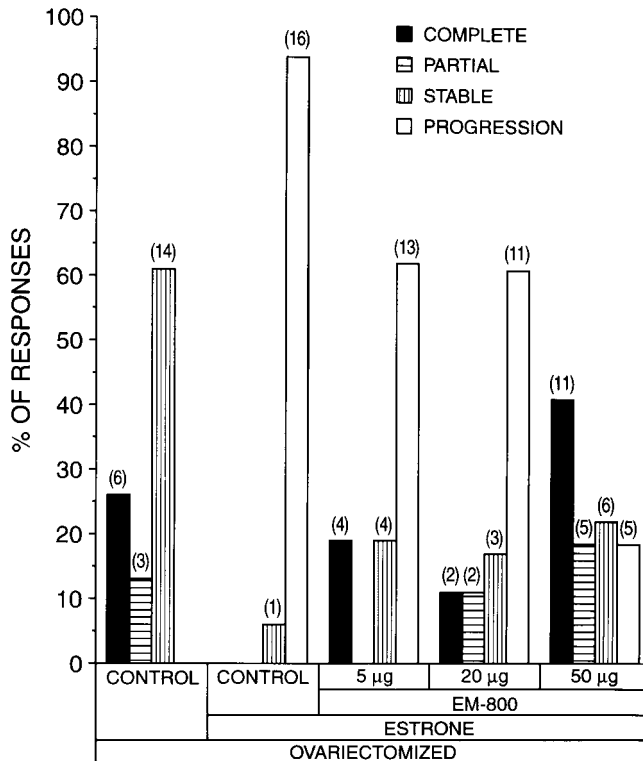


FIGURE 3 – Effect of treatment with the pure antiestrogen EM-800 on tumor response. Tumor responses were categorized as complete, partial, stable or progression as described in Material and Methods. Tumor response was evaluated either at week 40 or at the death of the animal. The control, E₁ and 5 µg, 20 µg and 50 µg EM-800 dose groups contained 23, 17, 21, 18 and 27 tumors at the start of treatment, respectively. The numbers in parentheses represent the number of tumors in each category.

to those observed in control OVX mice, thus indicating a near complete or complete inhibition of the effect of estrone by EM-800. Treatment with the lower daily oral dose of 5 µg of EM-800 caused a 59% inhibition of the stimulatory effect of estrone on ZR-75-1 tumor size at 40 weeks ($p < 0.01$), while a 75% inhibition was achieved with the 20 µg dose ($p < 0.01$).

It is of interest to examine the categories of responses achieved by the different treatments (Fig. 3). The 50 µg daily dose of the antiestrogen caused the disappearance (complete response) of 41% of tumors, while a complete response was seen in 26% of tumors in the non-treated ovariectomized controls. In animals that received the daily 5 µg and 20 µg doses of EM-800, complete responses were seen in 19% and 11% of tumors, respectively. A more than 50% regression of tumors (partial response) was observed in 0%, 11% and 19% of tumors of the animals treated with the daily 5 µg, 20 µg and 50 µg doses of EM-800, respectively. None of the tumors that showed a complete or partial response showed progression at a later time interval. A stable response (less than 50% decrease or less than 25% increase in tumor size) was observed in 19%, 17% and 22% of the tumors of animals that received the 5 µg, 20 µg and 50 µg of EM-800, respectively (Fig. 3).

Sixteen of 17 (94%) tumors showed progression during the 40-week period in animals receiving estrogen supplementation with estrone. One tumor was classified as stable, although invasion by a neighboring tumor made specific measurements more difficult. At 40 weeks, tumor size was measured at 580% above pretreatment value in this group. In animals treated with 5 µg EM-800 daily, 13/21 (62%) tumors showed progression to reach 322% above

pretreatment tumor size at 40 weeks (Fig. 4a). In animals receiving the 20 µg dose of EM-800, 11/18 (61%) tumors progressed to reach an average size of 215% above pretreatment value at the end of the experiment, while in the group of animals that received the daily 50 µg dose of the antiestrogen, only 5/27 tumors (19%) progressed after a stable response. It is also apparent from the data obtained that increasing doses of EM-800 not only decreased the number of tumors that progressed but also had a marked inhibitory effect on the size of the smaller number of tumors that developed. In fact, while the size of tumors in the control group supplemented with estrone increased to 580% above pretreatment values, an increase to only 57% above initial tumor size was observed for the 5 tumors that eventually progressed in the group of animals receiving the 50 µg daily dose of EM-800 (Fig. 4a).

None of the tumors that showed a complete response ($n = 11$) or that reached a partial response ($n = 5$) during the course of treatment with the highest dose of EM-800 showed progression at later time intervals. As can also be seen in Fig. 4b, the only tumors that showed progressions at 40 weeks in animals treated with the daily 50 µg dose of EM-800 were among those that showed a stable response at an earlier time interval. The median times to achieve complete and partial responses were calculated at 61 and 75 days, respectively. In those tumors developing in animals dosed with 50 µg EM-800 after a stable response, the median time to progression was calculated at 138 days (Fig. 4b).

At the end of treatment, uteri and vagina were weighed to determine the antiestrogenic effect of EM-800 in comparison with both the ovariectomized control (OVX) and OVX estrone-treated control groups. While the daily s.c. administration of 0.5 µg of estrone to ovariectomized mice increased uterine weight from 25 ± 2 mg to 112 ± 15 mg ($p < 0.01$), daily oral administration of 5, 20 and 50 µg of the antiestrogen inhibited estrone stimulation of uterine weight by 48% ($p < 0.01$), 84% ($p < 0.01$) and 93% ($p < 0.01$), respectively (Fig. 5a). There was no statistically significant difference between the OVX control and OVX plus estrone group receiving the daily 50 µg dose of EM-800. Comparable findings were observed on vaginal weight where the value increased from 33 ± 5 mg in ovariectomized animals to 60 ± 2 mg ($p < 0.01$) under treatment with estrone (Fig. 5b). The 5 µg and 20 µg doses of EM-800 caused 64% ($p < 0.01$) and 97% ($p < 0.01$) inhibitions of the stimulatory effect of estrone, while at the 50 µg daily dose, vaginal weight decreased to 25 ± 1 mg, a value 28% below the one measured in ovariectomized animals.

DISCUSSION

Our data show that the daily 50 µg oral dose of the pure antiestrogen EM-800 completely or near completely neutralized the stimulatory effect of estrone and decreased tumor size 50% below the initial value measured at the start of treatment. A similar effect was observed on uterine and vaginal weights where the estrogenic stimulation of estrone was completely abolished. The lower 5 µg and 20 µg daily doses of EM-800 led to intermediate inhibitory effects on the same parameters.

In the model used, EM-800 was administered to ovariectomized 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 intact, nonsupplemented animals. Due to 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 (Labrie *et al.*, data not shown). This results from inhibition of the negative feedback action of estrogens at the hypothalamic level.

Although adjuvant treatment with tamoxifen delays breast cancer recurrence, improves survival in early breast cancer and induces remission in patients with advanced disease, its benefits are limited by the development of tamoxifen resistance (Howell *et al.*,

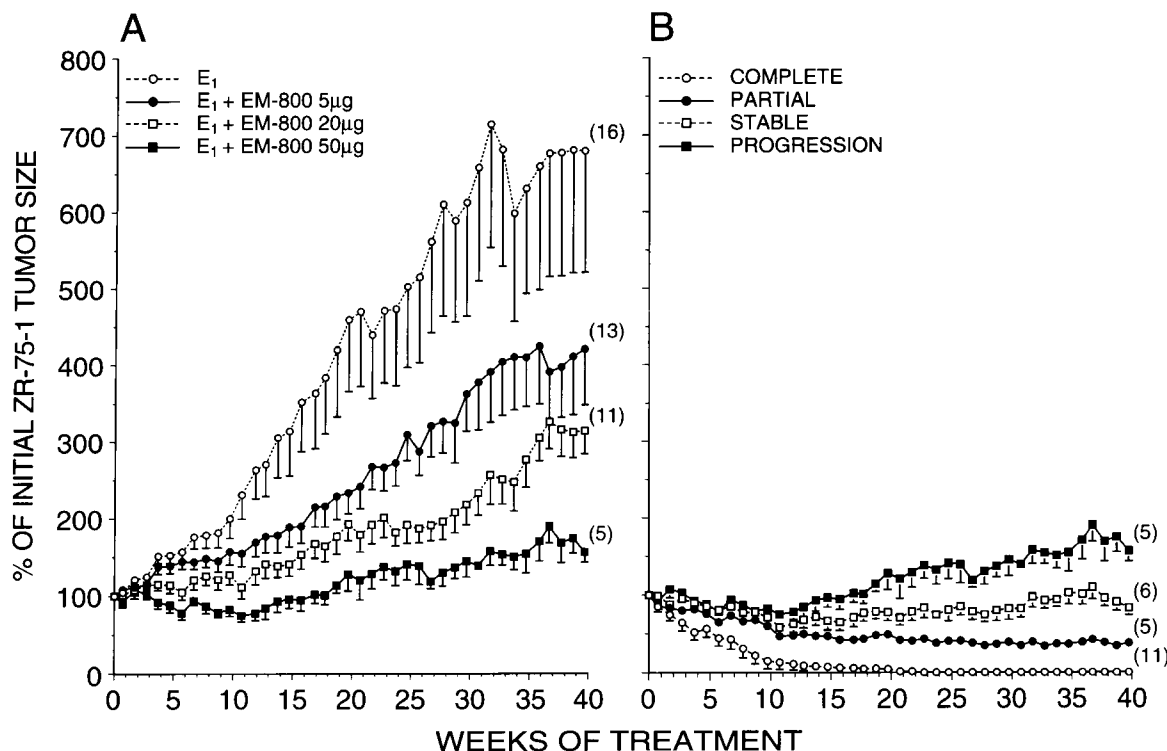


FIGURE 4 – (a) Mean size of tumors categorized as progressions over the course of treatment with E_1 alone and in combination with EM-800. Results are expressed as the percentage of pretreatment size (mean \pm SEM). Each data point represents tumors measured in live animals. The numbers in parentheses indicate the number of tumors classified as progressions in each group (see Fig. 3). (b) Mean tumor size as a function of category of response to treatment in mice dosed with estrone and 50 μg EM-800 for 40 weeks. Results are expressed as the mean \pm SEM calculated for the tumors that showed complete (n = 11), partial (n = 5), stable (n = 6) or progression (n = 5).

1996). Gottardis *et al.* (1988) have observed the acquired ability of tamoxifen to stimulate rather than to inhibit tumor growth. The observations that pure antiestrogens can inhibit the stimulatory effect of tamoxifen (Gottardis *et al.*, 1989; Osborne *et al.*, 1995) indicate that the stimulatory effect of tamoxifen upon long-term treatment is due to the intrinsic estrogenic activity of the compound.

In agreement with these data, we have observed that tamoxifen stimulates the growth of human ZR-75-1 xenografts in nude mice, while EM-800 has no stimulatory effect (Couillard *et al.*, 1998). Moreover, the stimulatory effect of tamoxifen on tumor growth was inhibited by simultaneous treatment with EM-800.

The novel nonsteroidal compound EM-800 and its metabolite EM-652 exert the most potent antagonistic effects of all compounds tested on estradiol-induced proliferation in T-47D, ZR-75-1 and MCF-7 human breast cancer cells in culture (Simard *et al.*, 1997a, data not shown). Furthermore, the absence of a stimulatory effect on basal cell proliferation shows that EM-652 and EM-800 are pure antiestrogens devoid of partial agonist activity in the 3 estrogen-sensitive human breast cancer cell lines used. The antiestrogenic activity of EM-652 and EM-800 on E_2 -induced cell proliferation in T-47D cells is at least 2 orders of magnitude more potent than tamoxifen, 2.5- to 3.6-fold more potent than OH-tamoxifen and 3.84-, 2.74- and 16.3-fold more potent than OH-Toremifene, ICI-182780 and ICI 164384, respectively (Simard *et al.*, 1997a). EM-800 was 46-fold more potent than Droloxifene in inhibiting E_2 -induced T-47D cell proliferation. As mentioned above, EM-800 and EM-652 have no estrogenic activity in the 3 breast cancer cell lines studied while OH-tamoxifen, Droloxifene and Toremifene cause a significant stimulation of ZR-75-1 and/or MCF-7 human breast cancer cell prolifer-

ation (Simard *et al.*, 1997a). The stimulatory effect of tamoxifen or OH-tamoxifen on human breast cancer cell growth has been reported previously by many laboratories under *in vitro* (Osborne *et al.*, 1985) as well as *in vivo* (Gottardis *et al.*, 1988) conditions.

The well-recognized intrinsic estrogenic activity of tamoxifen is likely to limit its success in the treatment of breast cancer in women (Furr and Jordan, 1984). Such an estrogenic action of tamoxifen in breast cancer in women is well supported by the tumor flare observed at start of therapy (Clarysse, 1985) as well as by the withdrawal response observed following arrest of tamoxifen in patients who progress under tamoxifen therapy (Howell *et al.*, 1992).

We have also observed that the new nonsteroidal antiestrogen EM-800 is devoid of any stimulatory estrogenic effect on alkaline phosphatase activity in human Ishikawa uterine carcinoma cells, whereas OH-tamoxifen is a relatively potent estrogen on this estrogen-sensitive parameter (Gauthier *et al.*, 1997; Simard *et al.*, 1997b). Furthermore, the stimulatory effect of OH-tamoxifen on alkaline phosphatase activity can be completely blocked by simultaneous exposure to the pure antiestrogens EM-800 or EM-139 (Labrie *et al.*, 1992), thus well supporting the suggestion that the effect of OH-tamoxifen in human uterine cells is mediated through activation of the estrogen receptor.

The appearance of uterine carcinoma in women treated with tamoxifen is not unexpected since tamoxifen has been shown to stimulate the growth of 2 human endometrial tumors implanted in nude mice (Clarke and Satyaswaroop, 1985; Gottardis *et al.*, 1988) as well as *in vitro* (Jamil *et al.*, 1991). Moreover, OH-tamoxifen has been shown to be potent, sometimes even more than E_2 itself, to stimulate the expression of progesterone receptors in the human

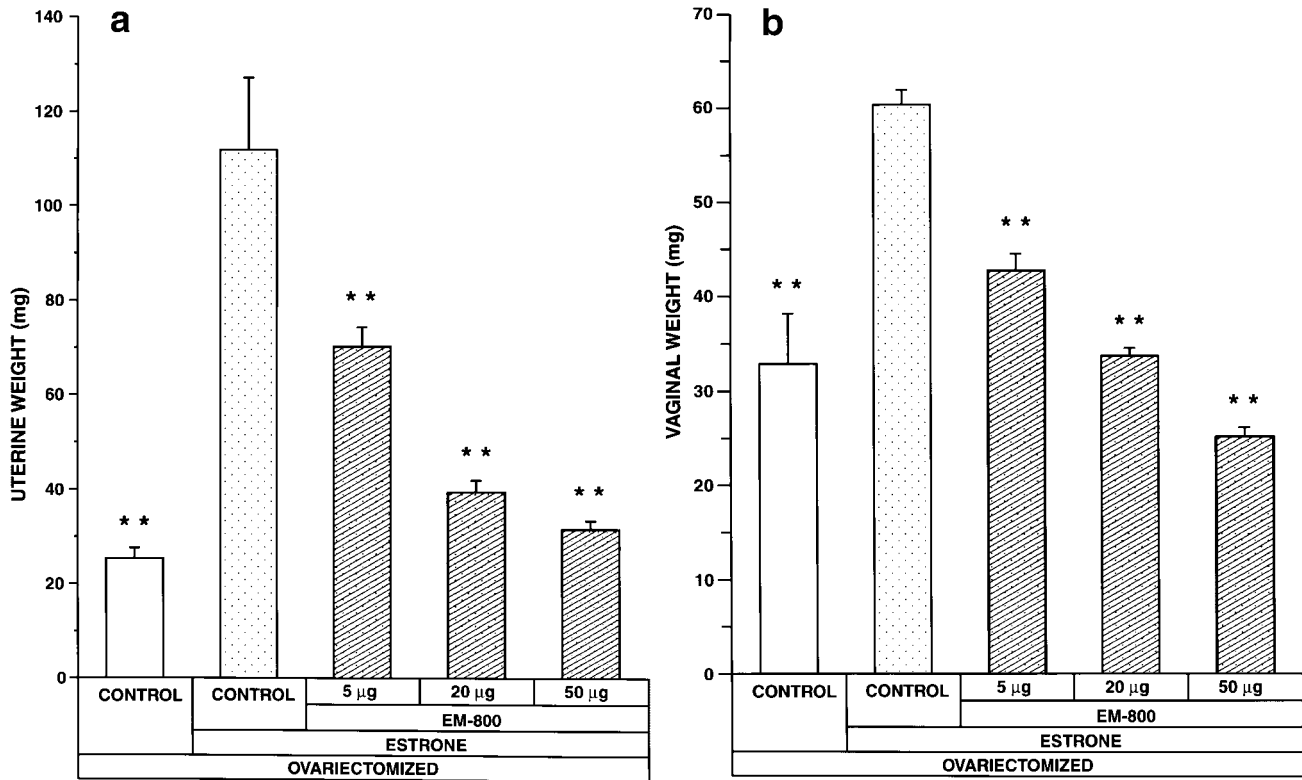


FIGURE 5 – Effect of treatment with the pure antiestrogen EM-800 at the daily oral doses of 5 µg, 20 µg or 50 µg for 40 weeks on uterine (a) and vaginal (b) weight in ovariectomized nude mice receiving a daily 0.5 µg s.c. dose of estrone. Mean uterine and vaginal weight in ovariectomized mice receiving the vehicle alone is shown for reference. **: $p < 0.01$ vs. E_1 -treated control.

Ishikawa endometrial cell line (Jamil *et al.*, 1991). It should be added that the relationship between estrogens and endometrial carcinoma is well recognized.

It seems reasonable to expect that the availability of a pure antiestrogen, in addition to avoiding the risk of inducing endometrial carcinoma, 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 so far be achieved in women suffering from breast cancer while the role of estrogens in this disease could not be satisfactorily evaluated.

Antiestrogens have so far been thought to have a cytostatic effect on breast cancer cells (Osborne *et al.*, 1995), most tumors becoming resistant to the antiestrogen (Osborne *et al.*, 1995). The present observation of a disappearance of 41% of tumors ($p <$

0.01) by daily treatment with 50 µg of EM-800 clearly suggests that efficient blockade of estrogens has a tumorigenic effect in addition to a tumorostatic effect on human breast cancer tumors. It can be mentioned that 26% of tumors disappeared upon careful histopathological evaluation in control OVX animals in the absence of estrogen supplementation.

So far, the response rates to all first line endocrine therapies have been similar at about one-third of an unselected population (Henderson, 1987). The response rate approaches 50% when the stable category is included. In view of the particularly high potency of this new antiestrogen and its highly specific antiestrogenic characteristics illustrated by its antiproliferative effect on estrogen-sensitive human breast cancer cells, 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 inhibitory response on breast cancer.

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