



Report

## A novel pure SERM achieves complete regression of the majority of human breast cancer tumors in nude mice

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### Summary

**Background.** The objective was to determine if EM-652, a novel selective estrogen receptor modulator (SERM) having highly potent and pure antiestrogenic activity in the mammary gland could cause complete regression of the majority of human breast cancer xenografts in nude mice.

**Methods.** Human breast cancer ZR-75-1 xenografts were used as model in nude mice.

**Results.** EM-652 not only prevented estrogen-induced tumor growth but it reduced tumor size to 20% of the pretreatment value. Complete disappearance of the tumors was observed in 65% (106/163) of tumors. No tumor progressed. Most importantly, 93% of the tumors which had become undetectable under EM-652 treatment did not reappear when exposed to estrogen challenge for 12 weeks, thus achieving an overall 61% cure rate.

**Conclusions.** The present data demonstrate that EM-652 is strongly cytotoxic or tumorocidal and not only cytostatic or tumorostatic in estrogen-sensitive breast cancer, thus changing the paradigm of a tumorostatic role of estrogen blockade established with tamoxifen. These findings support the use of such a compound for more efficient breast cancer prevention and therapy.

### Introduction

It is well recognized that the rate of tumor growth is the balance between cell proliferation and cell death [1, 2]. Hormonal therapy of breast cancer has generally been considered to be tumorostatic, thus implying that induction of cell death or apoptosis is not the predominant mechanism involved. In fact, the tumorostatic idea which implies an absence of cure with hormonal therapy was first proposed in the course of experiments performed with tamoxifen in human breast tumors in the athymic mouse model [3, 4]. Recently, however, there have been a number of studies showing that increased cell death accompanies endocrine manipulations in various animal models [2, 5–13]. There is thus increasing recognition that induction of apoptosis by antihormones is an important mechanism involved in the efficient treatment of hormone-sensitive tumors, although the possibility of

a complete disappearance of tumors or cure has not been generally recognized as an objective achievable with estrogen blockade.

Since the first step in the action of estrogens in target tissues is binding to the estrogen receptors (ERs) [14, 15], a logical approach for the treatment of estrogen-sensitive breast cancer is the use of antiestrogens that competitively bind to ERs and block estrogen action. In this context, tamoxifen has been widely used over the past decades and has shown important benefits in breast cancer therapy at all stages of the disease. Unfortunately, in patients with advanced disease, an important proportion do not respond and for those who initially respond, resistance to treatment rapidly develops in most cases [16]. The absence or loss of response to tamoxifen might be attributed to a suboptimal blockade of estrogen action or to the partial agonistic activity of the compound [17–19]. Because of these limitations of tamoxifen, major efforts have been

devoted to the development of new antiestrogens devoid of intrinsic agonistic activity [20–22]. Moreover, the lack or loss of tumor inhibition by tamoxifen could be explained by the inability of this compound to block ER activation by growth factors and other factors that act through the MAP kinase pathway at the AF-1 site of both ER $\alpha$  and ER $\beta$  [23–25].

EM-652 is a highly potent nonsteroidal pure estrogen antagonist of both alpha and beta ER subtypes in the mammary gland and uterus [23, 24, 26, 27]. In fact, EM-652 has the highest affinity of any known compound for the ER, including estradiol, 4-OHTamoxifen, and ICI 182,780 [28]. The objective of the present study is to determine the potential cytotoxic or tumorocidal effect of EM-652 in the well-characterized estrogen-sensitive ZR-75-1 human breast cancer xenograft model in nude mice.

## Materials and methods

### *Human ZR-75-1 breast cancer cells*

ER-positive ZR-75-1 human breast cancer cells obtained from the American Type Culture Collection (Rockville, MD) were cultured in phenol red-free RPMI-1640 medium. The cells were supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU penicillin/ml, 100  $\mu$ g streptomycin/ml, and 10% (v/v) fetal bovine serum and incubated under a humidified atmosphere of 95% air/5% CO<sub>2</sub> at 37°C. Cells were passaged weekly and harvested at 85–90% confluence using 0.083% pancreatin/0.3 mM EDTA.

### *Animals, tumor inoculation and study design*

Homozygous female nu/nu Br athymic mice (28–42 days old) were obtained from Charles River, Inc. (Saint-Constant, Québec, Canada). The mice (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 h of light and 12 h of darkness (lights on at 07:15 h). Cages, bedding, water and food (Agway Pro-Lab R-M-H Diet #4018) were autoclaved before use. Bilateral ovariectomy (OVX) was performed under isoflurane-induced anesthesia. At OVX, each mouse received a 0.5 cm subcutaneous silastic implant containing estradiol (E<sub>2</sub>) diluted with cholesterol at a ratio of 1:10. One week after OVX,  $2.7 \times 10^6$

ZR-75-1 cells (passage 92) in the logarithmic growth phase were harvested with 0.083% pancreatin/0.3 mM EDTA and inoculated subcutaneously in 0.1 ml RPMI-1640 medium + 30% Matrigel in the right flank of each mouse. After 2 weeks, the E<sub>2</sub> implants were replaced in all animals by estrone-containing (E<sub>1</sub>) implants (E<sub>1</sub>:chol, 1:25, w:w).

Tumor-bearing mice were randomly assigned to two groups of 30 tumors for the control group (OVX + E<sub>1</sub>) and 163 tumors for the group of animals with E<sub>1</sub> implants which also received the antiestrogen. Thereafter, E<sub>1</sub>-containing implants in the two groups were changed every 6 weeks.

### *Antiestrogen therapy and study design*

EM-652·HCl was synthesized in the Medicinal Chemistry Division of our laboratory. The antiestrogens were given to each animal at the daily oral dose of 500  $\mu$ g suspended in 0.2 ml 0.4% (w/v) methylcellulose for 287 days. Animals in the control group received 0.2 ml of the vehicle alone.

### *Tumor measurements and necropsy*

Two perpendicular diameters were recorded and tumor area (mm<sup>2</sup>) was calculated using the formula:  $(L/2)(W/2)\pi$ . The area measured on the first day of treatment was taken as 100%.

After 287 days of treatment, the remaining animals were sacrificed. To further characterize the effect of the antiestrogen, the uterus and vagina were immediately removed, freed from connective and adipose tissue and weighed.

### *Evaluation of tumorocidal action or 'cure'*

A large number of tumors disappeared during the course of study. To evaluate the cure rate induced by EM-652, mice with tumors that regressed completely and thus remained undetectable for four consecutive weeks continued treatment with estrone only (without the antiestrogen) for another 12 weeks. Tumors that did not reappear after 12 weeks of estrogenic challenge were considered 'cured' (Table 1). The tumors that reappeared and reached 4.9 mm<sup>2</sup> were retreated with EM-652.

### *Response criteria*

The best response of the tumors to daily administration of EM-652 was assessed during the course of the study

or at death of each animal, if death occurred during the course of the experiment. Complete regression identifies those tumors which became undetectable; partial regression corresponds to the tumors that regressed >50% of their original size; stable response refers to tumors that regressed ≤50% or progressed ≤50%; and progression indicates that tumor size increased more than 50% compared to original size.

*Statistical analyses*

The change in total tumor areas between days 1 and 287 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 treatment effects at 287 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 (Kruskal-Wallis) were therefore selected for the analyses. A posteriori pairwise comparisons of means were performed using the Tukey HSD test. The vagina weight was checked for normal distribution by Shapiro-Wilk *W*-test for goodness of fit after log linear transformation. Analysis of distribution of uterine weight values could not be fitted to normal distribution after any of the transformations that were attempted. Therefore, ranks were used to test the hypothesis. The effect of treatment on uterine and vaginal weights at time 287 days was analyzed by one-way ANOVA. A posteriori comparisons of the means were performed using the Tukey HSD test. The overall type 1 error rate ( $\alpha$ ) was controlled at 5% to declare significance of the differences. All calculations were performed using Jump Software (SAS Institute, Carry, NC).

**Results**

As illustrated in Figure 1, estrone alone (OVX + E<sub>1</sub>) caused a progressive increase in ZR-75-1 average tumor size that reached 570% of the initial value after 41 weeks of treatment. Treatment with EM-652 not only blocked estrogen stimulation of tumor growth but it reduced tumor size 80% below the pretreatment value. After only 7 weeks of treatment, EM-652 had already reduced average tumor size by 63% compared with start of treatment.

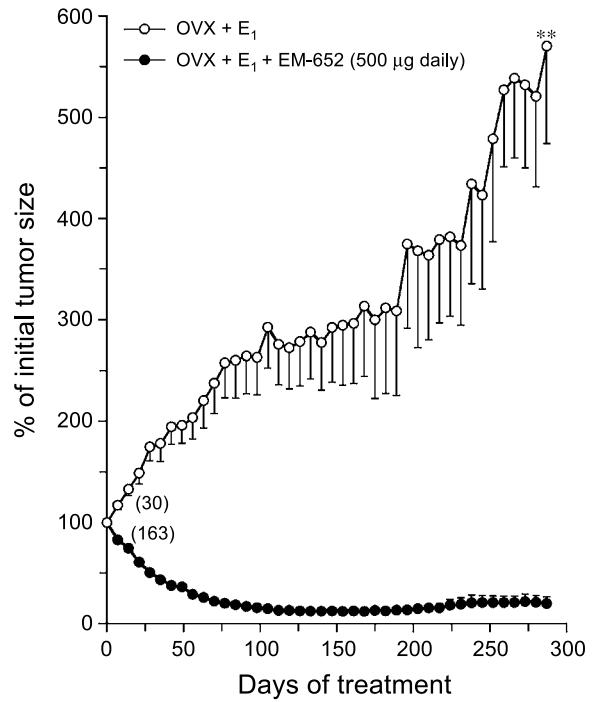


Figure 1. Effect of daily administration of EM-652 on the growth of human ZR-75-1 breast cancer xenografts in ovariectomized nude mice supplemented with estrone. Tumor size measured weekly is expressed as the percentage of initial tumor area. The average tumor size at the start of the study was 21.97 ± 1.52 mm<sup>2</sup> (range 4.71–40.42 mm<sup>2</sup>). Individual tumor areas calculated on day 1 of the experiment were assigned a value of 100%. All subsequent tumor sizes were expressed as a % of day 1 values (\*\* *p* < 0.01).

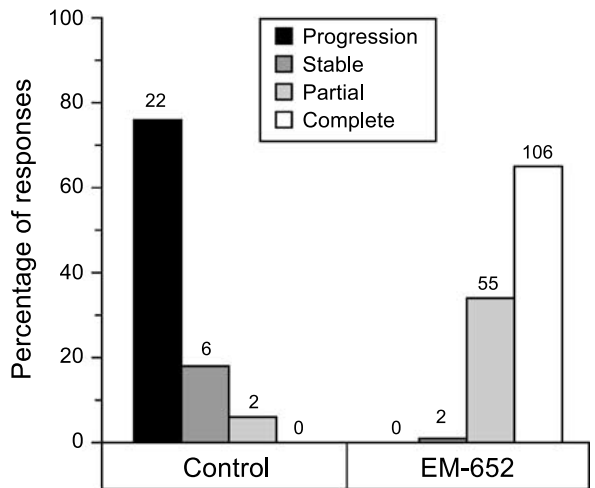


Figure 2. Best responses achieved during daily administration of EM-652 in human ZR-75-1 breast cancer xenografts in estrone-supplemented ovariectomized nude mice. The number of tumors in each category is indicated.

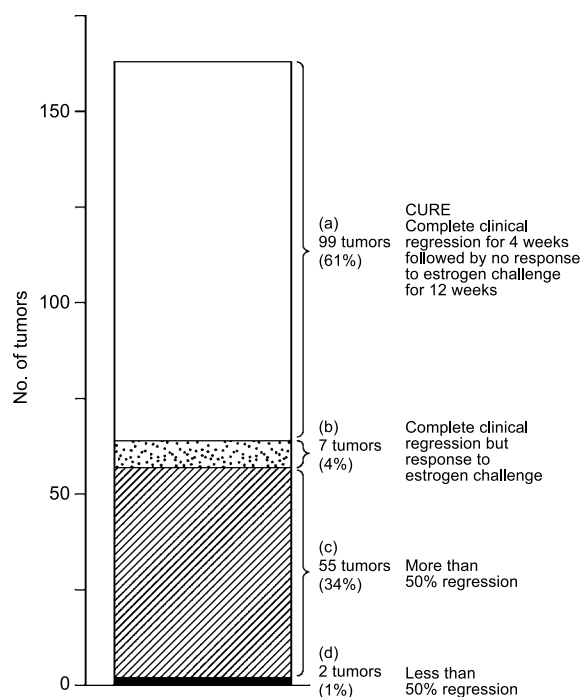


Figure 3. Percentages of tumors which (a) were cured or did not respond to the 12-week estrogen challenge after being non-detectable for 4 weeks (99 tumors, 61% of total); (b) become undetectable for 4 weeks but responded to the estrogen challenge (7 tumors, 4% of total); (c) regressed by more than 50% (55 tumors, 34% of total) or (d) regressed by less than 50% (2 tumors, 1%). No tumor continued to progress or showed progression as response; the total number of tumors was 163.

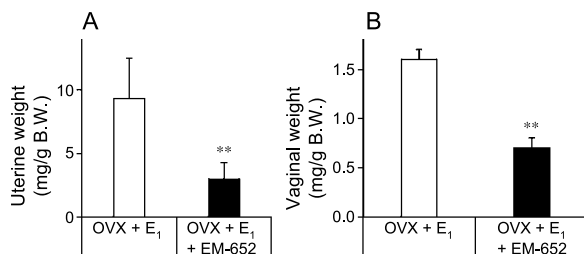


Figure 4. Effect of 41-week oral administration of EM-652 on uterine (A) and vaginal (B) weight in estrone-stimulated ovariectomized nude mice. Data are presented as mean  $\pm$  SEM ( $n = 7$  animals/group for estrone alone) and ( $n = 14$  animals/group for treatment with estrone and EM-652) (\*\*  $p < 0.01$ ).

It is of particular interest to analyze the categories of responses achieved during treatment with the antiestrogen. In OVX animals supplemented with estrone, 73% (22 of 30) of tumors progressed while no progression occurred in the animals who received EM-652 in addition to E<sub>1</sub>. In the antiestrogen-treated animals, complete, partial, and stable responses were achieved

in 65, 34, and 1% of tumors, respectively (Figure 2). It is remarkable that all tumors regressed with 65% of tumors achieving a complete regression, while 34% regressed more than by 50% and only two tumors regressed less than 50%. Only two tumors which had achieved a partial response and one stable response progressed during the course of the study.

Most importantly, 65% of the tumors in the group of EM-652-treated animals became undetectable for four consecutive weeks (106/163). Treatment with the antiestrogen was then stopped in these animals. A total of 95 mice were thus isolated and treated with estrone alone to test if the tumors which became undetectable under EM-652 treatment would reappear upon challenge with estrone. It is quite remarkable that only 7 of the 106 tumors which had become undetectable by palpation for 4 weeks reappeared under estrone challenge for 12 weeks (Figure 3). These seven tumors were retreated with EM-652, and a second inhibition of growth was observed in all cases, thus showing that no resistance to EM-652 had developed. This figure also shows that 55 tumors (34%) showed more than a 50% regression while only 2 tumors (1%) regressed less than 50%.

Treatment with the antiestrogen decreased uterine and vaginal weight by 68% and 56%, respectively (Figure 4).

## Discussion

The present study shows that during a 41-week treatment period, the antiestrogen EM-652 causes cure of 61% of human breast cancer tumors in nude mice. In fact, 65% of tumors became clinically undetectable during treatment and only 7% of these tumors reappeared following cessation of EM-652 and exposure to the stimulatory effect of the estrogen alone, thus showing an overall 61% cure rate. The disappearance of 61% of tumors during treatment with EM-652 clearly indicates that the antiestrogen induces cell death or apoptosis in ZR-75-1 human breast cancer cells and that efficient estrogen blockade achieved with a potent and pure antiestrogen is tumorocidal and not only tumorostatic as established previously with tamoxifen [3, 4].

Most interestingly, tumors which reappeared under estrone challenge alone still responded quickly to second treatment with the antiestrogen, thus showing that resistance to EM-652 had not developed as observed with tamoxifen [18, 29, 30]. Comparable

results have recently been achieved in men with localized prostate cancer where an absence of rise of prostatic-specific antigen (PSA) for at least 5 years after cessation of long-term combined androgen blockade indicates efficient control or most probably cure of the disease in 90% of cases [31]. In 10% of cases where PSA rose following cessation of long-term androgen blockade, re-starting combined androgen blockade led to a second PSA response, thus indicating that the cancer had remained sensitive to androgens, as observed in the present study for human breast cancer xenografts.

EM-652 is the most potent of all antiestrogens tested to inhibit the proliferation of MCF-7, ZR-75-1 and T-47D human breast cancer cells [32]. The present data show that EM-652 alone decreases tumor volume as efficiently as achieved in the complete absence of estrogens, namely in control OVX animals, thus confirming the pure antiestrogenic activity of the compound under *in vivo* conditions [29, 33]. It is of major interest that the addition of radiation therapy to complete blockade of estrogenic action by EM-652 has been found to further decrease tumor size and induce the disappearance of tumors to an even greater degree than achieved by OVX alone [33]. Similar findings were observed following the addition of cyclophosphamide to EM-652 [34]. Such data indicate that the mechanisms of apoptosis induced by estrogen blockade, radiotherapy and chemotherapy can be additive, thus offering the possibility of increasing the efficiency of breast cancer therapy.

Tamoxifen has been found to induce apoptosis and reduce tumor cell proliferation [2, 8, 12]. Moreover, the tamoxifen analogue idoxifene reduces Ki67 levels by about 30% in postmenopausal women with ER-positive tumors, although no effect could be seen on apoptosis [35]. Our previous data have shown that EM-652 exerts no agonistic effect on ZR-75-1 tumor growth either in *in vitro* or *in vivo* [32, 36] and is more potent than tamoxifen to inhibit estrogen stimulation. In fact, EM-652 does even block the stimulatory effect of tamoxifen on human breast cancer tumor growth in nude mice [36]. Moreover, of all the compounds tested, the novel nonsteroidal antiestrogen EM-652 exerts the most potent antagonistic effects on estradiol-induced proliferation in T-47D, ZR-75-1, and MCF-7 human breast cancer cells in culture [32].

Interestingly, a recent *in vivo* study that compared the inhibitory effect of seven antiestrogens in the nude mouse model bearing ZR-75-1 human breast tumors has shown that tamoxifen, idoxifene, ralox-

ifene, toremifene and GW 5638 show similar effects by inhibiting estrone stimulation by 60–80%. Droloxifene, on the other hand inhibited estrone stimulation by only 30% while EM-652 achieved the greatest inhibition of tumor size with a 95% inhibition of estrone stimulation [29]. In agreement with these preclinical data, positive results have been observed with EM-800, a prodrug of EM-652, in breast cancer patients who had failed tamoxifen therapy. In this trial, 44.2% of patients (19/43) showed positive responses for at least 3 months and 37.2% (16/43) for at least 6 months [25]. These clinical results, as well as a long series of *in vitro* and *in vivo* results, confirm that EM-652 is the most potent and a completely pure antiestrogen in breast cancer.

The present data strongly support the use of pure antiestrogens for breast cancer prevention and therapy. It should be mentioned that while exerting pure antiestrogenic action in the mammary gland and uterus [25, 28, 32, 37], EM-652 protects against bone loss and reduces serum cholesterol in the rat [38]. The present observation of the disappearance or cure of 61% of human breast cancer tumors strongly supports the interest of further studies with this compound for both the prevention and treatment of breast and uterine cancer.

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