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EM-652 (SCH 57068), a third generation SERM acting as pure antiestrogen in the mammary gland and endometrium ☆

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Abstract

Breast cancer is the most frequent cancer in women while it is the second cause of cancer death. Estrogens are well recognized to play the predominant role in breast cancer development and growth and much efforts have been devoted to the blockade of estrogen formation and action. The most widely used therapy of breast cancer which has shown benefits at all stages of the disease is the use of the antiestrogen Tamoxifen. This compound, however, possesses mixed agonist and antagonist activity and major efforts have been devoted to the development of compounds having pure antiestrogenic activity in the mammary gland and endometrium. Such a compound would avoid the problem of stimulation of the endometrium and the risk of endometrial carcinoma. We have thus synthesized an orally active non-steroidal antiestrogen, EM-652 (SCH 57068) and the prodrug EM-800 (SCH57050) which are the most potent of the known antiestrogens. EM-652 is the compound having the highest affinity for the estrogen receptor, including estradiol. It has higher affinity for the ER than ICI 182780, hydroxytamoxifen, raloxifene, droloxifene and hydroxytoremifene. EM-652 has the most potent inhibitory activity on both ERα and ERβ compared to any of the other antiestrogens tested. An important aspect of EM-652 is that it inhibits both the AF1 and AF2 functions of both ERα and ERβ while the inhibitory action of hydroxytamoxifen is limited to AF2, the ligand-dependent function of the estrogen receptors. AF1 activity is constitutive, ligand-independent and is responsible for mediation of the activity of growth factors and of the ras oncogene and MAP-kinase pathway. EM-652 inhibits Ras-induced transcriptional activity of ERα and ERβ and blocks SRC-1-stimulated activity of the two receptors. EM-652 was also found to block the recruitment of SRC-1 at AF1 of ERβ, this ligand-independent activation of AF1 being closely related to phosphorylation of the steroid receptors by protein kinase. Most importantly, the antiestrogen hydroxytamoxifen has no inhibitory effect on the SRC-1-induced ERβ activity while the pure antiestrogen EM-652 completely abolishes this effect, thus strengthening the need to use pure antiestrogens in breast cancer therapy in order to control all known aspects of ER-regulated gene expression. In fact, the absence of blockade of AF2 by hydroxytamoxifen could explain why the benefits of tamoxifen observed up to 5 years become negative at longer time intervals and why resistance develops to tamoxifen. EM-800, the prodrug of EM-652, has been shown to prevent the development of dimethylbenz(a)anthracene (DMBA)-induced mammary carcinoma in the rat, a well-recognized model of human breast cancer. It is of interest that the addition of dehydroepiandrosterone, a precursor of androgens, to EM-800, led to complete inhibition of tumor development in this model. Not only the development, but also the growth of established DMBA-induced mammary carcinoma was inhibited by treatment with EM-800. An inhibitory effect was also observed when medroxyprogesterone was added to treatment with EM-800. Uterine size was reduced to castration levels in the groups of animals treated with EM-800. An almost complete disappearance of estrogen receptors was observed in the uterus, vaginum and

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tumors in nude mice treated with EM-800. EM-652 was the most potent antiestrogen to inhibit the growth of human breast cancer ZR-75-1, MCF-7 and T-47D cells in vitro when compared with ICI 182780, ICI 164384, hydroxytamoxifen, and droloxifene. Moreover, EM-652 and EM-800 have no stimulatory effect on the basal levels of cell proliferation in the absence of E2 while hydroxytamoxifen and droloxifene had a stimulatory effect on the basal growth of T-47D and ZR-75-1 cells. EM-652 was also the most potent inhibitor of the percentage of cycling cancer cells. When human breast cancer ZR-75-1 xenografts were grown in nude mice, EM-800 led to a complete inhibition of the stimulatory effect of estrogens in ovariectomized mice while tamoxifen was less potent and even stimulated the growth of the tumors in the absence of estrogens, thus illustrating the stimulatory effect of tamoxifen on breast cancer growth. When incubated with human Ishikawa endometrial carcinoma cells, EM-800 had no stimulatory effect on alkaline phosphatase activity, an estrogen-sensitive parameter. Raloxifene, droloxifene, hydroxytoremifene and hydroxytamoxifen, on the other hand, all stimulated to various extent, the activity of this enzyme. The stimulatory effect of all four compounds was blocked by EM-800, thus confirming their estrogenic activity in human endometrial tissue. When administered to ovariectomized animals, EM-800 prevents bone loss, the effect on bone mineral density, trabecular bone volume, and trabecular separation being 5–10 times more potent than raloxifene. EM-800 lowers serum cholesterol and triglyceride levels in the rat as well as in women. In a Phase II study performed in patients with breast cancer showing failure on tamoxifen, 1 patient had a complete response while 5 patients had a partial response and stable disease for at least three months has been observed in an additional 13 patients for a total of 19 positive responses out of 43 evaluable patients (44.2%). No significant secondary effect related to the drug was observed. A phase 3 international clinical trial is currently being performed in tamoxifen failure patients where EM-800 (SCH 57050) is compared to Arimidex. The detailed information obtained at the preclinical level with EM-652 or EM-800 indicates that these orally active compounds are highly potent and pure antiestrogens in the mammary gland and endometrium while they prevent bone loss and lower serum cholesterol and triglyceride levels. Preclinical and clinical data clearly suggest the interest of studying this compound in the neoadjuvant and adjuvant settings and, most importantly, for the prevention of breast and uterine cancer in which settings they should provide additional benefits on bone and lipids.

Keywords: Pure antiestrogen; Breast cancer; Uterine cancer; EM-652; SCH 57068; EM-800; SCH 57050; Osteoporosis; Selective estrogen receptor modulator (SERM); Cholesterol; Triglycerides; Prevention; Risk reduction; Adjuvant; Neoadjuvant

1. Introduction

1.1. Breast cancer

Breast cancer is the most frequent cancer in women, with 176,300 new cases and 43,700 deaths predicted in the United States in 1999 [1]. Present therapies in breast cancer achieve meaningful clinical results in only 30–40% of patients, with response duration usually limited to 12–18 months [2–5]. Five-year survival in women with metastatic disease is still only 10–40%.

Among all risk factors, estrogens are well recognized to play the predominant role in breast cancer development and growth [6–9]. However, existing surgical or medical ablative procedures do not result in complete elimination of estrogens in women [10], due to the contribution of the adrenal glands that secrete high levels of dehydroepiandrosterone (DHEA) and DHEA-sulfate which are converted into estrogens in peripheral target tissues [11–13]. Considerable attention has thus been focused on the development of blockers of estrogen biosynthesis and action [14–20].

Since the first step in the action of estrogens in target tissues is binding to the estrogen receptor [21,22], a logical approach for the treatment of estrogen-sensitive breast cancer is the use of antiestrogens, or compounds which block the interaction of estrogens with their specific receptor. Until very recently, no agent with pure antiestrogenic activity under in vivo conditions has been available.

1.2. Tamoxifen

Tamoxifen, the antiestrogen most widely used for the treatment of women with breast cancer has shown clear clinical benefit in advanced breast cancer, its efficacy being comparable to that achieved with ablative and additive therapies [23]. In the first clinical studies initiated in 1969, tamoxifen was found to achieve remissions in advanced breast carcinoma similar to those observed following estrogen therapy but with fewer side effects [24]. Since then, because of its favorable safety profile and clinical efficacy comparable to other endocrine therapies, including oophorectomy and estrogens, tamoxifen has become the treatment of choice for patients with advanced or metastatic breast cancer [25–27]. This compound, however, is known to possess mixed estrogenic and antiestrogenic activities [19,23,28] which are species-, tissue-, cell-, and even gene-specific [29,30]. In support of the clinical evidence for the estrogenic activity of tamoxifen on human breast cancer growth [31,32], 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 [29,33–40]. Tamoxifen may act as an estrogen agonist more frequently than generally thought and this may explain some of the apparent paradoxes

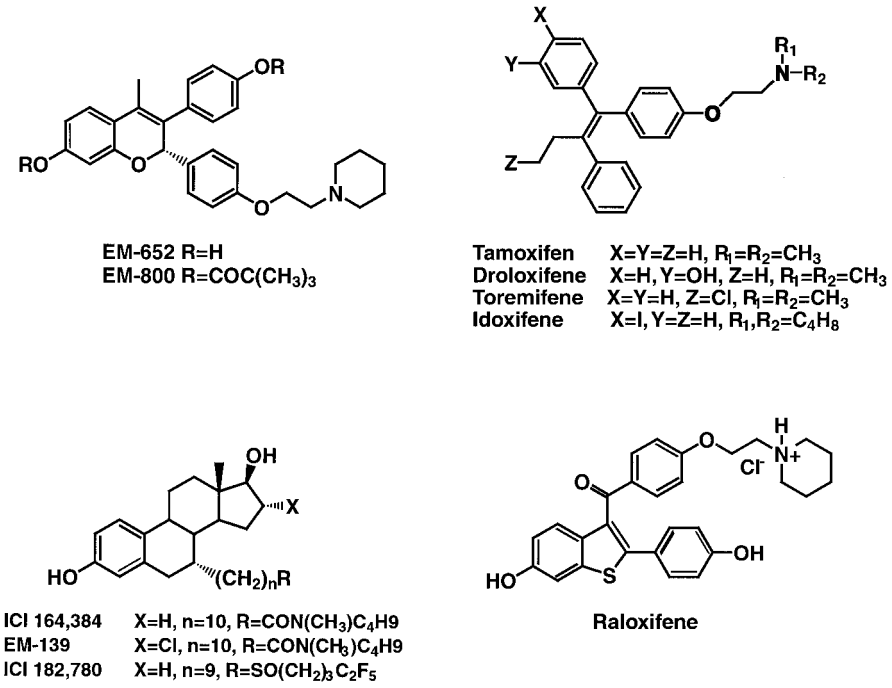

Fig. 1. Antiestrogens—molecular structures.

of endocrine treatments such as response to second endocrine therapy and withdrawal responses [27].

Additionally, while benefits of tamoxifen are observed on breast cancer in up to 40% of patients, the long-term use of this compound has recently been recognized as being associated with a significant increase in the incidence of endometrial carcinoma [41–55], an effect which is likely caused by the intrinsic estrogenic activity of the compound and possibly because of its genotoxic action on the DNA, by forming DNA adducts. The close analogs of tamoxifen, namely toremifene, Idoxifene and droloxifene, also possess estrogenic effects analogous to those of tamoxifen [56,57 data not shown].

2. Need for an orally active pure antiestrogen in the mammary gland and endometrium

Since clinical data suggest that long-term (5 years) tamoxifen adjuvant therapy is preferable to the short-term (2 years) use of the antiestrogen [58,59] and studies are in progress on the long-term use of tamoxifen as a chemo-preventive in breast cancer [54,60,61], it has become important to develop a pure antiestrogen to avoid the negative effects of the partial estrogenic activity of Tamoxifen and thus make available a compound having activities limited to the desired therapeutic action. The first class of pure antiestrogens obtained were 7α-substituted estradiol derivatives [5,14,16,18,19,62,63], especially ICI 164,384, EM-139, and ICI 182,780 (Fig. 1).

These compounds have been shown to possess pure and potent antiestrogenic activity in most well recog-

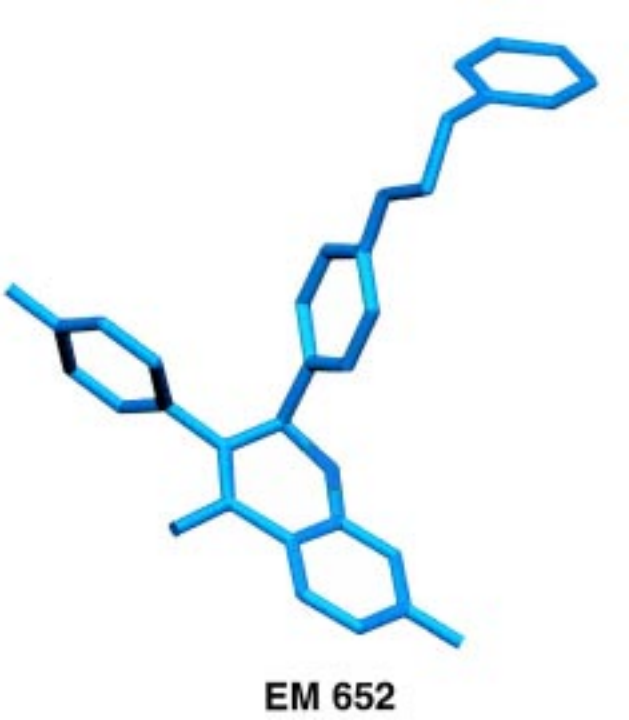

Fig. 2. Structure of EM-652 (SCH 57068).

Table 1
Comparison of the estrogen receptor affinity of a series of antiestrogens and related compounds with estradiol (E_2) and diethylstilbestrol (DES) in human breast cancer and normal human uterine cytosol[a]

	Breast Cancer				Uterus			
	ethanol		DMF		ethanol		DMF	
Compounds	K_i (nM) (max)	RBA	K_i (max)	RBA	K_i (max)	RBA	K_i (max)	RBA
E_2	0.138	100	0.113	100	0.120	100	0.181	100
DES	0.126	110	–	–	0.128	93.5	–	–
(S)-**6**(EM-652)	0.047	291	0.076	150	0.042	284	0.069	264
(R)-**6**(EM-651)	2.09	6.62	–	–	1.89	6.34	–	–
(S)-**1**(EM-800)	4.71	2.32	–	–	11.14	1.32	–	–
(R)-**1**(EM-776)	> 270	< 0.04	–	–	–	–	–	–
ICI 164,384	4.60	3.00	1.53	7.46	2.33	5.15	1.76	10.3
ICI 182,780	7.63	1.81	0.755	15.1	–	–	0.668	27.2
(Z)-4-OH-Tamoxifen	0.249	43.8	–	–	0.346	43.8	–	–
Tamoxifen	11.9	0.92	–	–	34.4	0.92	–	–

[a] Incubations were performed at room temperature for 3 h using 100 μL of cytosol, 100 μL of [^{3}H]E_2 (5 nM E_2, final) and 100 μL of the indicated unlabelled compounds leading to final concentrations of 3.3% ethanol or 2.5% dimethylformamide (DMF). The apparent inhibition constant (K_i) and relative binding affinity (RBA) values were calculated as described [73,223]. The apparent inhibition constant K_i values were calculated according to the following equation: $K_i = IC_{50}/(1 + S/K)$ where S represents the concentration of labelled E_2 and $K$is the K_D value of E_2 (0.14 nM) for the estrogen receptor. RBA values were calculated as follows: RBA = IC_{50} of E_2/IC_{50} of tested compound ×100 [56].

nized in vitro and in vivo systems, including human breast cancer cells [14,16,18,19,64,65]. The 7α-alkyl estradiol derivative ICI 164384, however, has been found to possess some estrogenic agonistic activity in guinea pig uterine cells [66,67]. Furthermore, both OH-tamoxifen and ICI-164384 can stimulate CAT activity in MCF-7 cells transfected with a pS2-tkCAT fusion gene [68]. Moreover, such 7α-alkyl estradiol derivatives are difficult to synthesize and their bioavailability by the oral route is very low, thus necessitating parenteral administration. We therefore concentrated our efforts on the synthesis of non-steroidal compounds having oral activity in order to overcome this difficulty.

In order to facilitate large-scale purification, EM-800 (SCH 57050), the bipivalate derivative of EM-652 was synthesized. EM-800 is rapidly transformed into EM-652 in intact cells and following in vivo administration. The other derivative currently used in our studies is EM-652.HCl (SCH 57068.HCl). In an effort to develop an orally active agent, EM-652 was synthesized (Fig. 2). As will be discussed later, the active compound EM-652 derived from EM-800 or EM-652.HCl behaves as a highly potent and pure antiestrogen in human breast and uterine cancer cells in vitro as well as in vivo in nude mice [56,69–72].

3. Binding characteristics to the estrogen receptors α and β

The estrogen receptor affinity of EM-652, the active drug of EM-800, was first measured in human breast cancer and normal human uterine cytosol as described [56,73]. As measured by competition studies in human breast cancer tissue, the affinity of EM-652 ($K_i = 0.047 \pm 0.003$ nM, RBA = 291, relative to 17β-estradiol set at 100) studied in the presence of ethanol was 2.9 higher

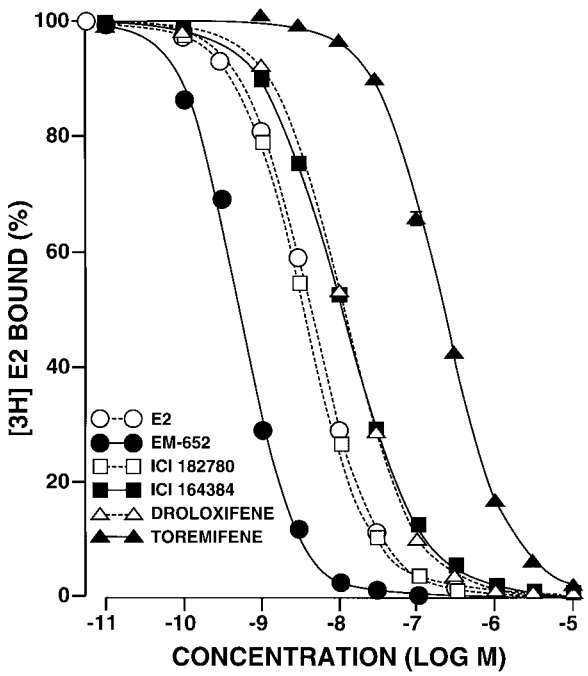

Fig. 3. Effect of increasing concentrations of EM-652, E2, ICI 182780, Droloxifene, ICI 164384, and Toremifene on [^{3}H] 17β-estradiol binding to the rat uterine estrogen receptor. The incubation was performed with 5 nM [^{3}H] 17β-estradiol (E_2) for 2 h at room temperature in the presence or absence of the indicated concentrations of unlabeled compounds [224].

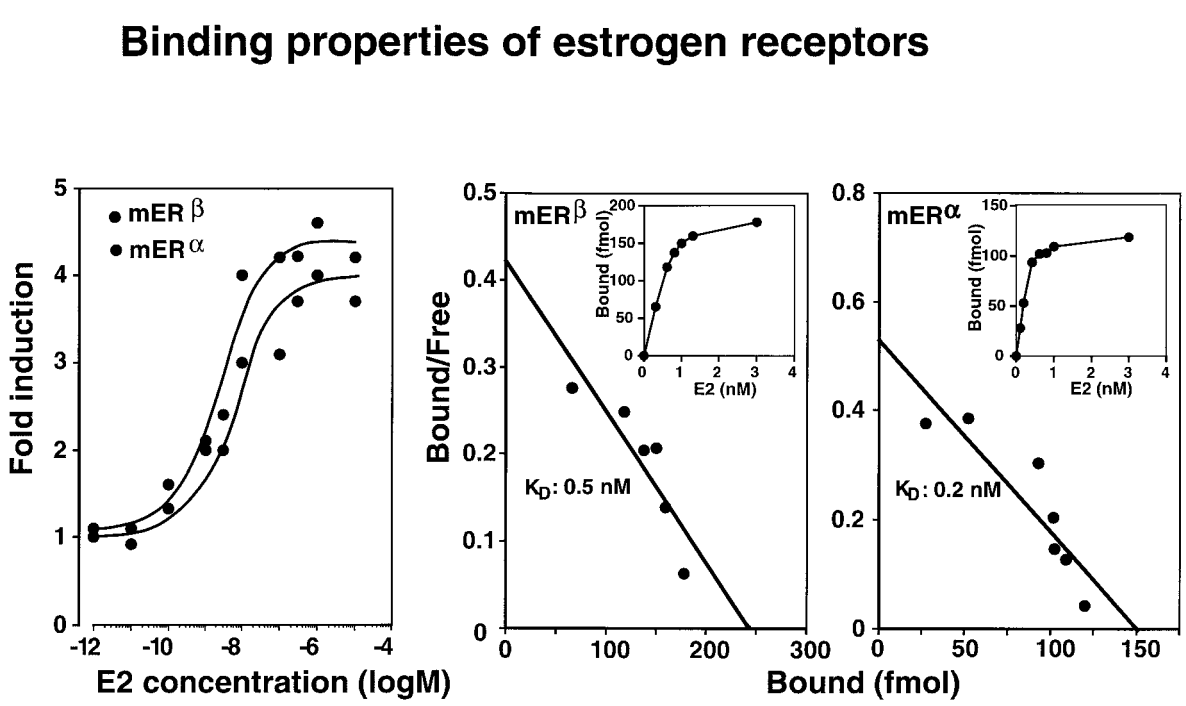

Fig. 4. Dose-response and binding properties of mERα and mERβ. (A) Cos-1 cells were transfected with 500 ng mERβ (open circles) or mERα (closed circles) expression vectors and 1 μg vitA$_2$-ERE-TKLuc and then incubated for 12 h with increasing concentrations of E_2 as indicated. (B) Specific binding of [2,4,6,7-3H]-17β-estradiol ([^{3}H]E_2) to mERβ was determined using receptors generated from rabbit reticulocyte lysates. Binding was determined over a concentration range of 0.01–3 nM [^{3}H]E_2 in the absence or presence of a 200-fold excess of unlabeled E_2. The saturation plot is shown in the inset, and results were plotted by the method of Scatchard. Each point was determined in triplicate in each experiment, and the above results are representative of at least two separate experiments. (C) Specific binding to mERα using the conditions described in panel B [65].

than that of estradiol itself (RBA = 6.62). Similar results were obtained on the human uterine estrogen receptor (Table 1). It can be seen in the same table that ICI 182,780 has about 10 times lower affinity than EM-652 to displace [^{3}H]E_2 from the human estrogen receptor while (Z)-4-OH-Tamoxifen is about 6 times less potent under the experimental conditions used. The new antiestrogen EM-652 thus shows the highest affinity for the human estrogen receptor of all the compounds tested [56] (Table 1).

It can be seen in Fig. 3 that EM-652 is 7- to 8-fold more potent than E2 and ICI 182780 in displacing [^{3}H]E_2 from the rat uterine estrogen receptor (IC_{50} values of 0.52, 4.13, and 3.59 nM for EM-652, E_2, and ICI 182780, respectively). ICI 164384 and Droloxifene are 21-fold less potent than EM-652 while Toremifene is 400 times less potent than EM-652.

Over the past decade, all the studies on the elucidation of the molecular events underlying the mode of ER action as well as the antiestrogen-designed therapy have focused on the ERα identified and cloned several years ago [21,74,75]. Recently, a second estrogen receptor, designated ERβ, has been described and shown to share common structural and functional characteristics with ERα [65,76,77]. Based on amino acid sequence comparison, ERβ shares with ERα the same modular structure composed of six domains (A–F) [78]. Domain C, which contains the two zinc fingers responsible for DNA binding, is the most conserved followed by domain E, responsible for ligand binding, homodimerization and nuclear localization. Domain E also contains a ligand-dependent activation function (AF-2) involved in *trans*-activation by the ERs. A second activation function, AF-1, resides in the A/B domain and acts in a ligand-independent manner [79–81].

Both ERs recognize a specific estrogen response element (ERE) composed of two AGGTCA motif half-sites configured as a palindrome spaced by three nucleotides [65]. ERα has also been shown to interact with a number of coregulators via the AF-2 domain, and these protein–protein interactions promote transcriptional regulation of target genes [82–85]. Following cloning of mouse ERβ [65], comparison could be made of the activity of ERα and ERβ and measurement could be made of the affinity of the two ERs for various ligands, especially, antiestrogens.

We first tested the activity of both receptors in the presence of increasing E_2 concentrations using the vitA2-ERE-TKLuc reporter in Cos-1 cells. Comparison of the dose–response curves of Fig. 4A shows a shift of approximately 4-fold of the E_2 concentration required to achieve half of the maximal

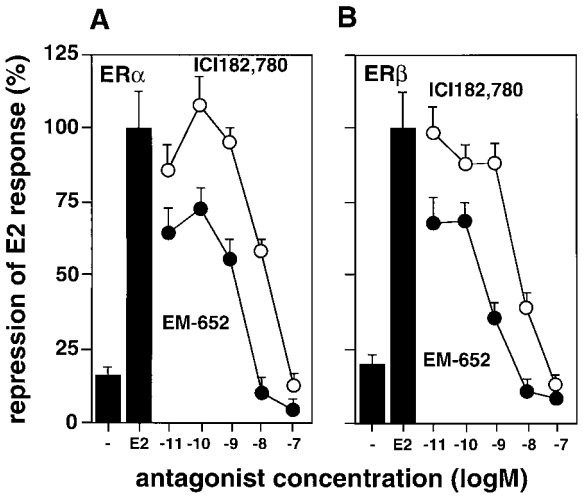

Fig. 5. Dose–response of antagonists on ERα- and ERβ-mediated transactivation. Comparison of the dose–responses of the antagonists in the presence of 10 nM E_2 on the transcriptional activity of ERα (A) and ERβ (B) using the vitA_2ERETKLuc reporter in COS-1 cells. Results represent the mean ±SEM of three separate experiments and are expressed as percentages of the maximal induction by E_2 alone (set arbitrarily at 100%; filled bars) for the two ERs. The untreated ERα and ERβ basal levels are also shown [105].

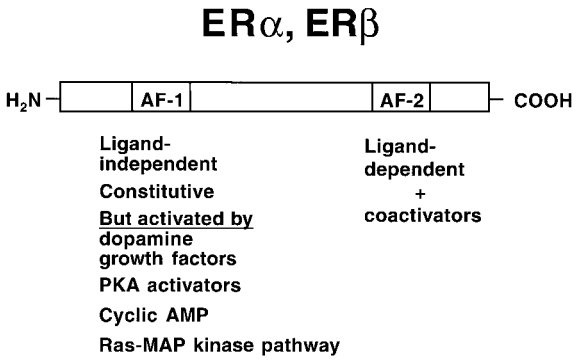

Fig. 6. Schematic representation of the activation functions 1 and 2 of ERα and ERβ. AF-1 is ligand-independent but is activated by dopamine, growth factors, cyclic AMP, MAP kinase, PKA activators and RAS. AF-2 on the other hand, is activated by estrogenic compounds. EM-652 blocks both AF-1 and AF-2 completely while OH-Tamoxifen blocks AF-2 only.

level of induction between the two receptors, the ERα being more sensitive to E_2.

The above-indicated results already suggested that ERβ may have lower affinity for E_2 than mERα. To verify if the difference in E_2 responsiveness was due to a difference in ligand binding, we performed a binding analysis on both mERβ and mERα. [^{3}H]E_2 was used to conduct binding studies with mERβ, and results were plotted by the method of Scatchard. As shown in Fig. 4B, this analysis yielded an average dissociation constant (K_d) of 0.5 nM for E_2 when performed on ERβ prepared from rabbit reticulocyte lysates. This value is comparable to that obtained for the rat ERβ, which was reported to be 0.6 nM [76]. On the other hand, we obtained an average K_d of 0.2 nM for mERα (Fig. 4C), which is well within the range of previously published determinations for the cloned human receptor [86]. Therefore, this slightly reduced affinity of mERβ for E_2 may provide an explanation for the shift in E_2 responsiveness indicated by the dose-response curves (Fig. 4A).

To further evaluate the potency of various antiestrogens, we compared their dose-dependent inhibition of E_2-induced ERα and ERβ activity using vitA_2ERETKLuc in COS-1 cells (Fig. 5). When compared to ICI182,780, EM-652 was highly effective, achieving a complete blockade of the E_2-induced effect of ERα (Fig. 5A) and ERβ (Fig. 5B) at concentrations of 10^{-8} M and above. Comparison of the apparent IC_{50} values showed that under the conditions used, EM-652 was more potent in repressing ERα activity (IC_{50} = 2 nM) than ICI182,780 (IC_{50} = 20 nM). Both antiestrogens were more effective to inhibit ERβ than ERα function with IC_{50} values of 0.4 nM and 8 nM for EM-652 and ICI182,780, respectively. In addition, lower concentrations of EM-652 in the 10^{-10}–10^{-11} M range contributed already to a 25–30% reduction in the E_2 response of both ERs, and even when added at 10^{-13} M, EM-652 already showed a 20–25% repression (data not shown).

4. EM-652 inhibits both AF-1 and AF-2 functions of ERα and ERβ

As mentioned above, the two ERs share many functional characteristics based on their well conserved modular structure (Fig. 6). AF-2 is responsible for estrogen-dependent activation through recruitment of coactivator proteins including members of the steroid receptor coactivator (SRC) family [85,87–93]. On the other hand, AF-1 activity is constitutive and ligand-independent [79–81].

In addition to the classical hormone activation pathway, a number of steroid receptors including ERα and β have been shown to be activated by non steroidal agents (Fig. 6) including dopamine, growth factors and PKA activators [65,94–98].

4.1. EM-652 inhibits RAS-induced transcriptional activity of ERα and ERβ

Potential phosphorylation of serine 118 in human ERα [96,99,100] and serine 60 in mouse ERβ [65] through activation of the Ras-MAPK pathway has been shown to further maximize the E_2 response of both estrogen receptors. To investigate whether EM-652 could efficiently block this effect, we used the wild-type H-Ras and its dominant active form H-Ras^{V12} in

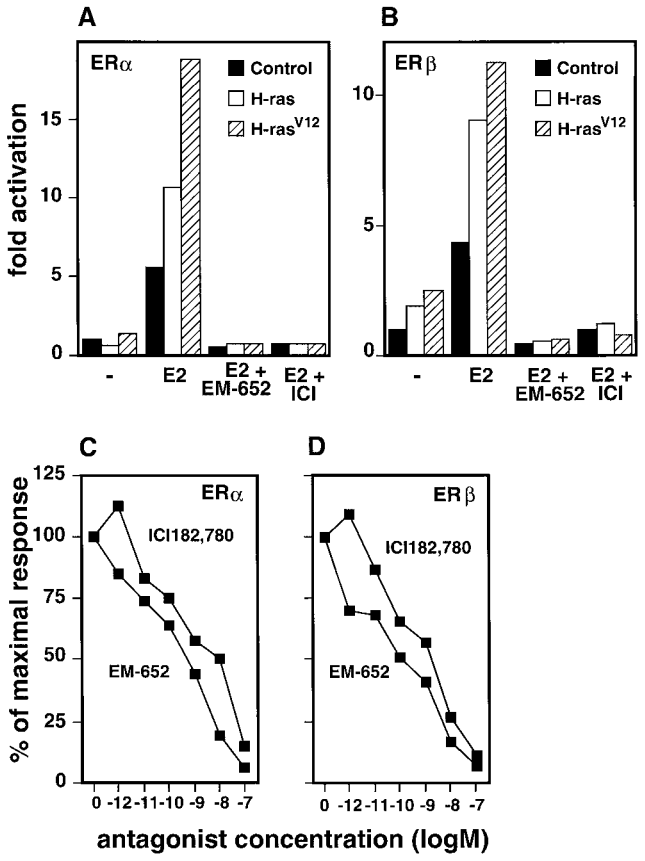

Fig. 7. EM-652 blocks the Ras-induced ERα and ERβ transcriptional activity. (A) COS-1 cells were cotransfected with 1 μg vitA$_2$ERETKLuc and 500 ng pCMX-ERα in the presence or absence of 1 μg Ha-Ras or Ha-Ras^{V12} expression plasmids. The cells were then grown in the presence or absence of 10 nM E_2 or 100 nM of EM-652 or ICI 182,780 (ICI). The basal activity of ERα in the absence of estradiol was set arbitrarily at 1.0. (B), same as in (A), except that ERβ expression vector was used. (C). Dose responses of EM-652 (filled squares) and ICI 182,780 (open squares) in the presence of 10 nM E_2 on ERα activity in COS-1 cells transfected with vitA$_2$ERETKLuc reporter and Ha-Ras^{V12} expression plasmid. The maximal induction by E_2 alone was set arbitrarily at 100%. (D) same as in (C) except that ERβ expression vector was used [105].

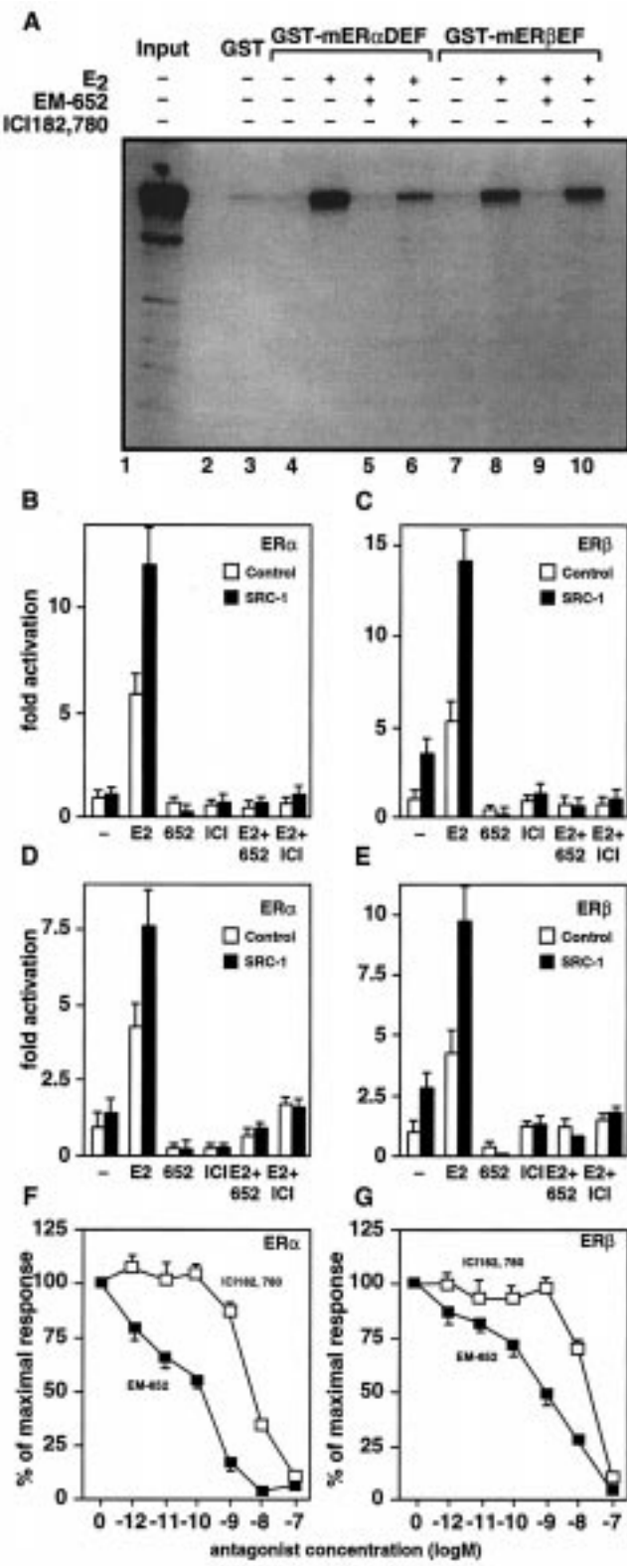

Fig. 8. EM-652 blocks the estrogen and SRC-1-stimulated AF2 activity of ERα and ERβ. (A) GST pull-down experiments. The purified fusion proteins were incubated with labeled SRC-1 in the absence (lanes 3 and 7) or presence of 5 nM E_2 (lanes 4–6 and 8–10) in addition to a 100-fold excess of EM-652 (lanes 5 and 9) and ICI 182,780 (lanes 6 and 10). The input lane (lane 1) represents 20% of the total amount of labeled SRC-1 used in each binding reaction. An equivalent amount of protein was used in the sample containing only GST (lane 2). (B) COS-1 cells were cotransfected with 1 μg vitA$_2$ERETKLuc and 500 ng pCMX-ERα in the presence or absence of 1 μg SRC-1 expression plasmid. Cells were incubated with or without 10 nM E_2 or 100 nM antagonist as indicated. Results are expressed as fold response over basal levels set arbitrarily at 1.0. (C), same as in (B), except that ERβ expression vector was used. (D) and (E), same as in (B) and (C) respectively, except that pS2Luc reporter and HeLa cells were used in transfections. (F), dose response of EM-652 (filled squares) and ICI 182,780 (open squares) in the presence of 10 nM E_2 on ERα activity in COS-1 cells transfected with vitA$_2$ERETKLuc reporter and SRC-1 expression plasmid. The maximal induction by E_2 alone was set arbitrarily at 100%. (G), same as in (F) except that ERβ expression vector was used [105].

our transfection experiments, as indicated in Fig. 7. As observed previously [65,100], the addition of H-Ras contributed to increase the activity of ERα in the presence of E_2, with an even stronger response when H-Ras^{V12} was used (Fig. 7A). These inductions by both Ras forms were completely abolished with the addition of EM-652 in the medium, as with ICI 182,780, suggesting that EM-652 is effective in blocking the AF-1 activity of ERα. The same experiment was also conducted on ERβ where H-Ras and H-Ras^{V12} augmented the E_2 response in a similar manner (Fig. 7B). Again, EM-652 and ICI 182,780 abolished the Ras effect on ERβ in the presence of E_2. Interestingly, we observed a ligand independent effect of Ras on ERβ basal activity where a 2–3-fold induction occurred with H-Ras^{V12} (Fig. 7B). On the other hand, no effect of Ras was seen on basal levels of ERα. The Ras induction of unliganded ERβ was blocked by EM-652 and ICI 182,780 (data not shown). We were also interested to test whether EM-652 was efficient in blocking ER responsiveness on a natural promoter. The pS2 promoter has been extensively studied in respect to its ERα mediated regulation [101]. We previously showed that ERβ can also modulate transactivation of a reporter gene driven by the pS2 promoter in HeLa cells, and that the E_2 response was potentiated by H-Ras [65]. The effects of Ras on liganded ERα and β activities are completely abrogated by EM-652 (data not shown). Dose response analyses were also performed to further evaluate the potency of EM-652 to inhibit the effect of Ras on ER activities in the presence of E_2. EM-652 was slightly more effective than ICI 182,780 in blocking H-Ras^{V12} inductions of ERα and ERβ, especially at lower concentrations (Fig. 7C and D).

4.2. EM-652 blocks SRC-1 induced activity of both ERα and ERβ

The co-activator SRC-1 has been shown to interact with and promote the transcriptional activity of a number of nuclear receptors including ERα [85,102]. More recently, we have demonstrated that SRC-1 also stimulates ERβ activity through a direct interaction with its ligand-binding domain (LBD) where the AF-2 domain resides [65]. We took advantage of this effect of SRC-1 to study whether EM-652 could block the E_2-activated AF-2 function of ERα and ERβ.

We first generated glutathione-S-transferase (GST) fusion proteins with the E and F domains of mERβ (GST-mERβ EF) and domains D–F of mERα (GST-mERα DEF) for use in GST-pull down experiments (Fig. 8A). GST-mERβ EF and GST-mERα DEF were expressed in *E. coli*, purified with GST-Sepharose and incubated with [^{35}S] methionine labeled SRC-1. As shown in Fig. 8A, the LBD of mERα interacted weakly with SRC-1 in the absence of E_2 (lane 3) whereas addition of E_2 caused an increase in interaction between the two proteins (lane 4). Both EM-652 (lane 5) and ICI 182,780 (lane 6) efficiently blocked the ligand-dependent SRC-1 interaction, with a stronger effect for EM-652. A similar inhibition of the E_2-dependent interaction between SRC-1 and the LBD of ERβ was also observed whereas ICI182,780 was less efficient (see Fig. 8A) lanes 7–10). We also demonstrate that the stimulatory effect of SRC-1 on the E_2 response of both ERs in COS-1 cells was completely abolished with the addition of EM-652 in the medium as did ICI 182,780 at the concentration used (Fig. 8B, C). Furthermore, as observed with Ras (see above), SRC-1, under the present experimental conditions, enhanced the basal activity of ERβ but not that of ERα in the absence of ligand. This ligand-independent effect of SRC-1 on ERβ was blocked by EM-652. Similar results were obtained using HeLa cells transfected with a pS2Luc reporter construct (Fig. 8D, E).

Dose response analyses were also performed to further evaluate the potency of EM-652 to inhibit the potentiating effect of SRC-1 on ER activities in the presence of E_2. EM-652 was very effective in blocking SRC-1 potentiation of ligand-dependent ERα and ERβ transcriptional activities with apparent IC_{50} values of 10^{-10} M and 10^{-9} M, respectively (Fig. 8F and G). ICI 182,780 was less potent to inhibit the SRC-1 induction of ERα and ERβ activities with an IC_{50} value of 10^{-8} M for both receptors.

The present study describes the molecular action of EM-652, the active metabolite of EM-800, on ER transcriptional functions. We present evidence that EM-800, and its active metabolite EM-652, act as pure estrogen antagonists on ERα and ERβ transcriptional activities. This pure antiestrogenic profile is of primary importance in endocrine-based breast cancer therapy, since the objective, as mentioned earlier, is to develop a compound having both activities, while the widely available antiestrogen currently available, Tamoxifen, acts as a mixed agonist–antagonist on ER function and does not inhibit the AF-1 function. Besides a relatively good clinical record in inducing remission of ER-positive breast cancer and in post-surgical adjuvant therapy, resistance to tamoxifen, a phenomenon likely due to its intrinsic agonist properties, does occur and tumor progression ensues in most of the patients [23].

The potency of EM-652 to inhibit ER function was even more dramatic when the E_2 response was maximized through activation of AF1 and AF2 domains of ERs by Ras and SRC-1, respectively. Phosphorylation of Ser^{118} triggered by the Ras-MAPK pathway has been described for ERα and shown to further increase its E_2-stimulated transcriptional activity [100]. Ras also activates liganded-ERβ presumably through phos-

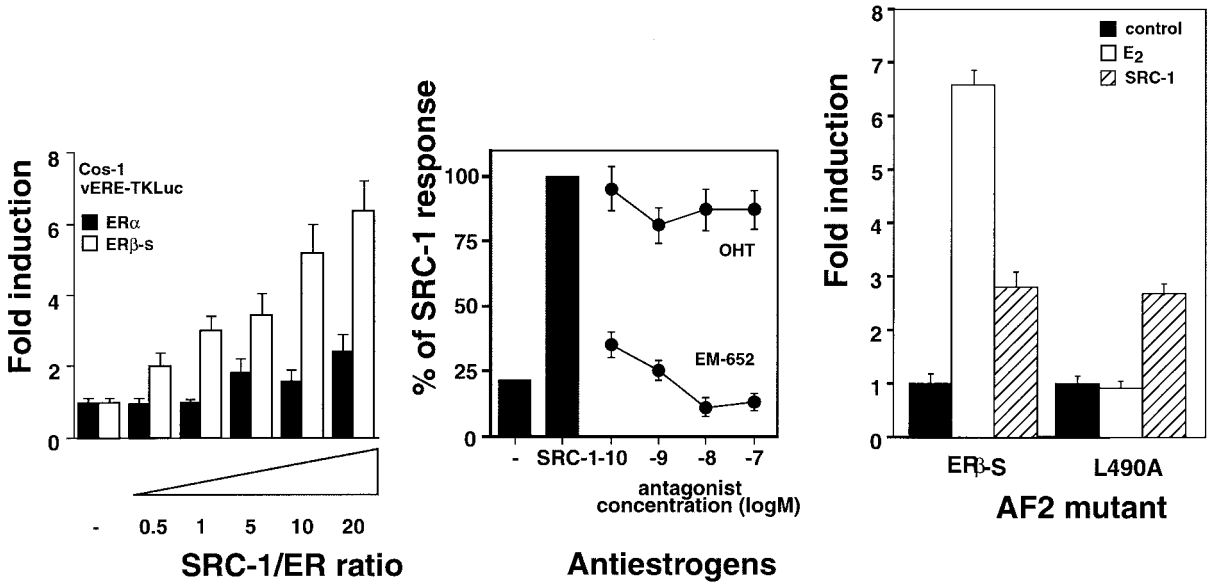

Fig. 9. Basal ERβ transcriptional activation by SRC-1 is AF-2 independent. (A) Dose dependent activation of ERα and β by SRC-1 in absence of E_2. Cos-1 cells were transfected with ERETKLuc reporter along with ERα or ERβ and increasing amounts of SRC-1 expression plasmids. Luciferase activities were normalized with β-gal expression and results are expressed as fold response over the basal levels (−) and represent the mean ± SEM of three independent experiments. (B) Pure antiestrogen EM-652 but not the mixed antagonist 4-OH-tamoxifen (OHT) inhibits basal ERβ transcriptional activation by SRC-1. Cos-1 cells were transfected with ERETKLuc along with equivalent amounts of ERβ and SRC-1 expression plasmids and incubated with increasing amounts of OHT or EM-652 prior to be assayed for luciferase activity. The maximal induction by SRC-1 alone (solid bar) was defined as 100%. Basal level in absence of SRC-1 is indicated by an open bar. (C) Basal activity of an ERβ AF-2 mutant is induced by SRC-1. Cos-1 cells were transfected with ERETKLuc reporter and equivalent amounts of ERβ or ERβ L509A AF-2 mutant and SRC-1 (filled bars) expression plasmids. Cells were then treated with 10 nM E_2 (shaded bars) or left untreated (open and filled bars) for 16 h prior to harvest. Results are plotted as fold induction over basal levels [109].

phorylation of Ser^{60} [65]. Here we show that EM-652 strongly inhibited the E_2-induced ERα and β activities triggered by either Ras or its dominant active form Ras^{V12}. We observed a similar pattern with SRC-1. SRC-1 is well known as a general coactivator for steroid receptors and shown to up-regulate ERα-stimulated transcription [85,103]. More recently, we demonstrated that SRC-1 interacts with ERβ and stimulates its transcriptional activity [65]. This interaction occurred with the LBD of both ERs [65,103]. Again, EM-652 was very potent in fully abolishing the E_2 response of ERα and ERβ enhanced by SRC-1. These effects were not cell- or promoter-specific as demonstrated with the pS2 promoter in HeLa cells. Hence, EM-652 can be regarded as a pure antagonist that acts on both activation domains of the ERs.

Interestingly, both Ras and Ras^{V12} induced the activation of transcription of ERβ in the absence of E_2. Such ligand-independent activation of Ras was not observed with ERα [100], although it was reported with EGF treatment [96]. A similar pattern of activation of ERβ but not ERα was also observed with SRC-1. Our previous work [65] has shown that the SRC-1-induced ligand-independent activation of ERβ was not blocked by OHT, which exerts an inhibition of ER limited to AF-2 [101], suggesting that SRC-1 might interact with other regions of the receptor. A possible target region for such an interaction might be contained within the amino-terminal region of ERβ as ICI 182,780 and EM-652 inhibit the ligand-independent effect of Ras and SRC-1.

4.3. EM-652 blocks the recruitment of SRC-1 at AF-1 of ERβ

The ligand-independent activation of AF-1 is presumed to be closely related to phosphorylation of steroid receptors by cellular protein kinases [104].

4.3.1. SRC-1 induces AF-2-independent ER transcriptional activation

Our previous observations that SRC-1 could stimulate ERα and β activity in absence of ligand prompted us to further investigate the mechanisms underlying this effect [105]. In absence of E_2 or other exogenously added stimulatory agents, SRC-1 increased in a dose-dependent manner the transcriptional activity of ERβ in transfected Cos-1 cells (Fig. 9A). This effect was not cell- nor reporter-specific since a similar increase in ERβ activity was detected on various reporters such as pS2Luc and ERE3TKLuc and in other cell lines

including HeLa and 293 T (data not shown). Interestingly, ERα was less sensitive than ERβ in terms of ligand-independent SRC-1 activation since a large excess of SRC-1 was required to reach an observable effect on ERα activity (Fig. 9A). Thus, the balance between the cellular content of SRC-1 and ER isoforms may contribute to discriminate between activation of unliganded ERα and β.

Based on the previous observation that SRC-1 could interact with steroid receptors in a ligand- and AF-2-independent manner [106–108], we tested whether AF-2 was necessary to mediate the SRC-1 effect on basal ERβ activation using the differential ability of mixed agonist/antagonists and pure antiestrogens to block AF-1 and AF-2 functions. EM-652, the active metabolite of EM-800, was previously identified as a very potent and pure antagonist of ERα and β transcriptional functions whereas 4-hydroxytamoxifen (OHT) only blocked AF-2 activity of both ERs [65]. As shown in Fig. 9B, EM-652 strongly impaired the SRC-1 mediated basal ERβ activity in Cos-1 cells while OHT treatment minimally decreased ligand-independent activation of ERβ. The same pattern of inhibition was obtained in HeLa cells with the pS2Luc reporter (data not shown). These data suggest that AF-2 is not required for the E2-independent activation of ERβ by SRC-1. To test this possibility further, we used an ERβ AF-2 deficient mutant ($ER\beta^{L509A}$) that is transcriptionally inactive in the presence of E_2 (Fig. 9C). SRC-1 could still activate $ER\beta^{L509A}$ in absence of ligand (Fig. 9C), thus demonstrating that the observed transcriptional effect of SRC-1 on unliganded ERβ occurred in an AF-2 independent fashion.

We also assessed the effect of phosphorylation of the ERβ AF-1 in regulating interaction with SRC-1 in vivo using transfection experiments. Treatment of transfected cells with PD98059, a selective inhibitor of MAPK activation, completely abrogated the SRC-1 mediated activation of unliganded ERβ, while the use of staurosporin, which inhibits protein kinase C, had no significant effect [109]. The interaction between ERβ and SRC-1 in absence of hormone was also demonstrated in vivo and shown to be influenced by factors known to change the phosphorylation status of nuclear receptors.

Our observations suggest that Ser^{106} and Ser^{124} are both required in vivo to fully recruit SRC-1. In addition, when cells were treated with factors known to activate Ras, such as EGF or IGF-1 (data not shown), the in vivo interaction between SRC-1 and ERβ was also enhanced, thus mimicking the results obtained in the presence of activated Ras.

This study demonstrates for the first time that phosphorylation of the AF-1 domain of a member of the nuclear receptor superfamily enhances the recruitment of a steroid receptor coactivator (SRC-1) and provides a molecular basis for ligand-independent activation of ERβ via the MAPK cascade.

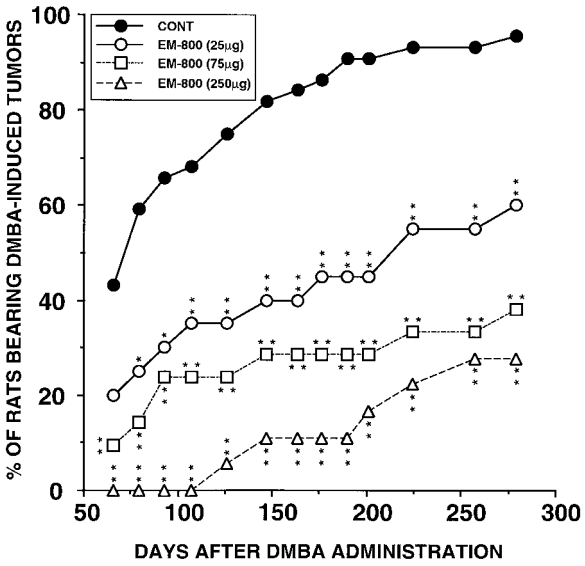

Fig. 10. Effect of daily oral administration of 25, 75, or 250 μg EM-800 on the number of animals who developed palpable mammary carcinoma induced by DMBA throughout the 279-day observation period. Data are expressed as percentage of the total number of animals in each group, $^{*}P < 0.05$; $^{**}P < 0.01$ vs control [222].

SRC-1 has been described as a coactivator that interacts and enhances the transcriptional activity of a number of nuclear receptors in a ligand- and AF2-dependent manner [85]. Based on three different approaches, enhancement of ERβ activation by SRC-1 in absence of ligand was found to be independent of AF-2. Very importantly, the partial antiestrogen OHT had no appreciable effect on SRC-1-induced unliganded ERβ activity while the pure antiestrogen EM-652 completely abolished this effect. This observation strengthens the need for pure antiestrogens in breast cancer therapy where all aspects of ER-regulated gene expression, including coactivator-mediated hormone-dependent as well as hormone independent activation pathways, must be regarded as direct targets for antiestrogen action. In fact, the absence of blockade of AF-2 by OH-TAM could explain why the benefits of Tamoxifen observed up to 5 years become negative at longer times of treatment and why resistance to Tamoxifen develops (Fig. 6).

5. Inhibition of the development and growth of DMBA-induced mammary tumors in the rat

5.1. Prevention of estrone-stimulated development of dimethylbenz(a) anthracene-induced mammary carcinoma in the rat

7,12-dimethylbenz(a)anthracene (DMBA)-induced

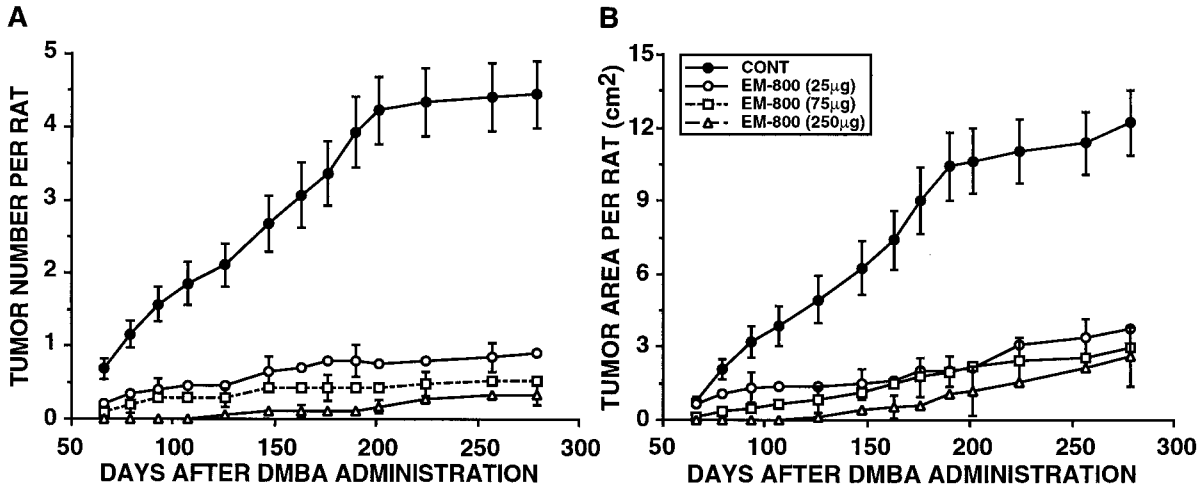

Fig. 11. Effect of daily oral administration of 25, 75, or 250 μg EM-800 on average tumor number per animal (A) and on average tumor size per rat (B) throughout the 279-day observation period. Data are presented as means ± SEM [222].

mammary carcinoma in the rat is a widely used animal model to study the factors which control hormone-sensitive breast cancer in women. In fact, the development and growth of these tumors are particularly sensitive to the stimulatory action of estrogen and prolactin [6,110–122].

An ideal antiestrogen should exert a highly potent inhibitory effect on breast cancer without showing any adverse effects on the endometrium or serum lipids and bone metabolism. We have thus investigated the effect of the new pure antiestrogen, EM-800 [56,65,70,123–125], on the development of mammary carcinoma induced by DMBA and the effect of such treatment on the serum lipid profile as well as on bone mass in the female rat.

As illustrated in Fig. 10, nine months after DMBA administration, 95% of control animals had developed palpable mammary carcinoma. In contrast, treatment with increasing doses of EM-800 caused a progressive inhibition of tumor development ($p < 0.0001$, for both the Fisher's exact test and the logistic regression), the incidence being reduced to 60%, 38%, and 28%, respectively. However, the difference between EM-800 doses is not statistically different. It is of interest to see in Fig. 11A that mean tumor number per animal was markedly decreased from 4.5 ± 0.5 tumors in the control group to 0.9 ± 0.2 ($p < 0.0001$), 0.5 ± 0.2 ($p < 0.0001$) and 0.3 ± 0.1 ($p < 0.0001$) tumors in the groups of rats treated with the antiestrogen. There is no statistically significant difference between the 3 groups treated with EM-800. In addition, the mean tumor area per animal was reduced from 12.2 ± 1.33 cm^2, to 3.76 ± 0.78 cm^2 ($p < 0.0001$), 2.94 ± 1.07 cm^2 ($p < 0.0001$) and 2.58 ± 1.21 cm^2 ($p < 0.0001$) following the same treatments (Fig. 11B).

Antiestrogens have been found to suppress tumorigenesis induced by chemical carcinogenic agents in the rat [114,117,120,126–128]. The present data clearly show that EM-800 not only significantly reduces the percentage of rats bearing DMBA-induced tumors, but also decreases tumor number per animal who has developed tumors during treatment with EM-800. Such data indicate the potential chemopreventive action of this compound in breast cancer. It is also of interest to note that the tumor size reached in the rats treated with EM-800 was smaller than that of control animals, a finding apparently in contrast with the data obtained with tamoxifen in the same animal model. In fact, Fendl and Zimniski [129] reported that the tumors that developed in rats treated with tamoxifen displayed a higher growth rate than the tumors in the control group. A strict comparison would, however,

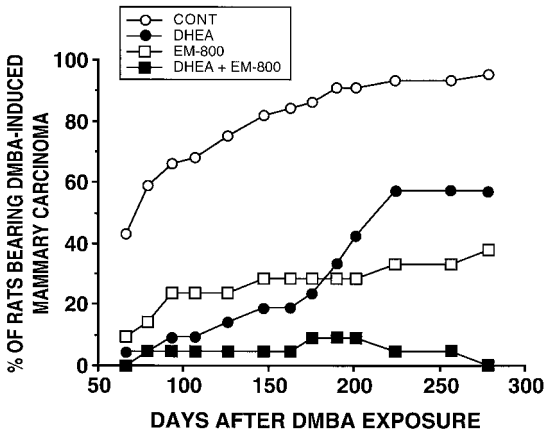

Fig. 12. Effect of treatment with DHEA (10 mg, percutaneously, once daily) or EM-800 (75 μg, orally, once daily) alone or in combination for 9 months on the incidence of DMBA-induced mammary carcinoma in the rat throughout the 279-day observation period. Data are expressed as percentage of the total number of animals in each group [124].

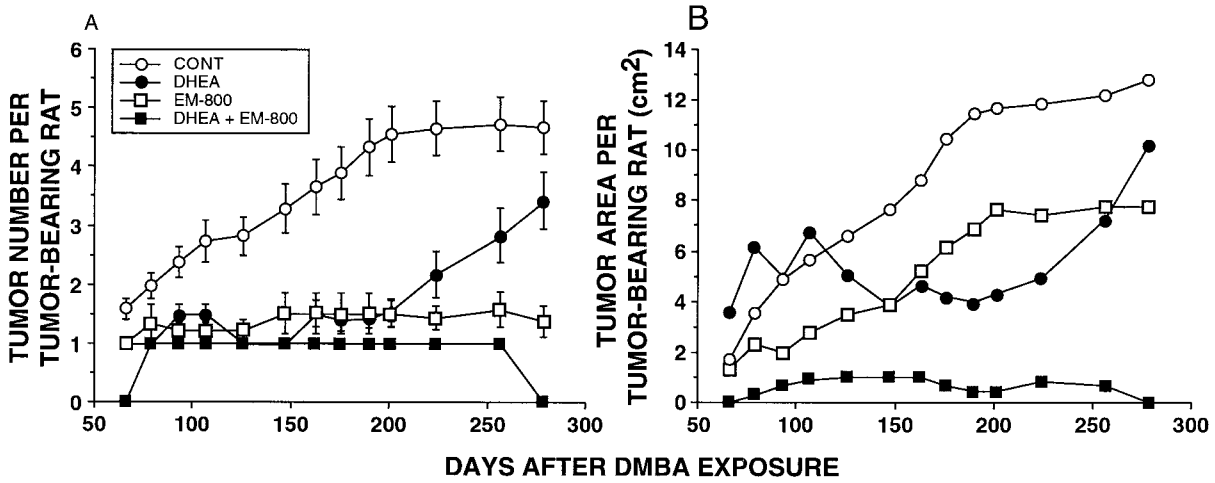

Fig. 13. Effect of treatment with DHEA (10 mg, percutaneously, once daily) or EM-800 (75 μg, orally, once daily) alone or in combination for 9 months on average tumor number per tumor-bearing animal (A) and on average tumor size per tumor-bearing rat (B) throughout the 279-day observation period. Data are expressed as the means ± SEM [124].

require a parallel evaluation of the two compounds in the same study.

The effects of EM-800 on serum lipids will be described later in this review.

5.2. Inhibition by EM-800 of estrone-stimulated growth of DMBA-induced mammary carcinoma in the rat—combination with DHEA

Since antiestrogens [15,17,127,128,130] as well as DHEA [122], independently, can inhibit the development of DMBA-induced mammary carcinoma, we have studied the potential benefits of combining the new antiestrogen EM-800 and DHEA on the development of mammary carcinoma induced by DMBA in the rat. Since the maintenance of bone density and the lipid profile are a main concern at menopause and during antiestrogen therapy, these parameters have been measured.

As illustrated in Fig. 12, 95% of control animals developed palpable mammary tumors by 279 days after DMBA administration. Treatment with DHEA or EM-800 partially prevented the development of DMBA-induced mammary carcinoma and the incidence was thus reduced to 57% ($p < 0.01$) and 38% ($p < 0.01$), respectively. Interestingly, combination of the two compounds led to a significantly higher inhibitory effect than those achieved by each compound alone ($p < 0.01$ versus DHEA or EM-800 alone). In fact, the only two tumors which developed in the group of animals treated with both compounds disappeared before the end of the experiment.

Treatment with DHEA or EM-800 decreased average tumor number per tumor-bearing animal from 4.7 ± 0.5 tumors in control animals to 3.4 ± 0.7 (NS) and 1.4 ± 0.3 ($p < 0.01$) tumors/animal, respectively, while no tumor was found at the end of the experiment in the animals who received both drugs ($p < 0.01$ versus the three other groups) (Fig. 13A). One of the two tumors which later disappeared was present from day 79 to day 201 following DMBA administration while the other tumor was palpable from day 176 to day 257. It can be seen in Fig. 13B that DHEA or EM-800 alone decreased average tumor area per tumor-bearing animal from 12.8 ± 1.3 cm^2 at the end of the experiment to 10.2 ± 2.1 cm^2 (NS) and 7.7 ± 1.8 cm^2 (NS), respectively, while the combination treatment resulted in a zero value ($p < 0.01$ versus the three other groups). The two tumors which developed in the group of animals treated with both DHEA and EM-800 did not grow larger than 1 cm^2. It should be mentioned that the real values of average tumor area as well as the average tumor number per tumor-bearing animal in the control group should be higher than the values presented in Fig. 13, since many rats had to be sacrificed before the end of the experiment because of the excessive size of tumors. The values measured at time of sacrifice were thus used as such in the calculations made at later time intervals in order to minimize a bias in the control group which, in any case, remained significantly above the other groups.

It has been observed that androgens exert a direct antiproliferative activity on the growth of ZR-75-1 human breast cancer cells in vitro and that such an inhibitory effect of androgens is additive to that of an antiestrogen [131,132]. Similar inhibitory effects have been observed in vivo on ZR-75-1 xenographts in nude mice [15,71]. Androgens have also been shown to inhibit the growth of DMBA-induced mammary carcinoma in the rat, this inhibition being reversed by the

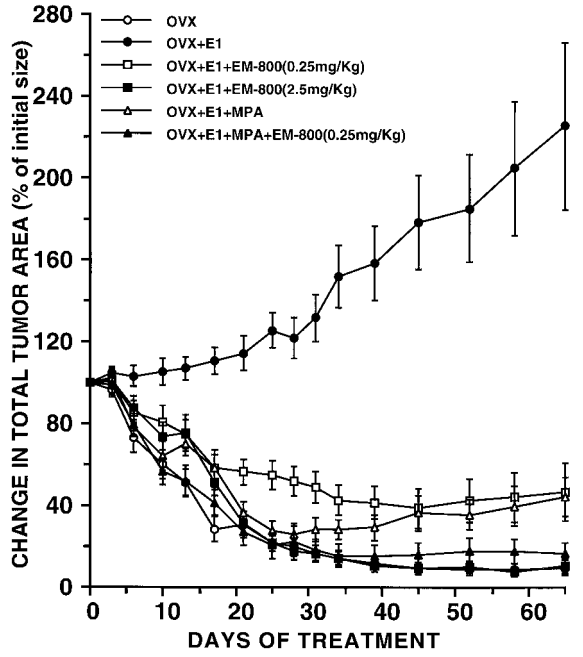

Fig. 14. Effect of 65-day-treatment with the antiestrogen EM-800 at the doses of 0.25 and 2.5 mg per kg body weight (orally, once daily) or medroxyprogesterone acetate (MPA, 1 mg s.c., twice daily) or the combination of EM-800 (0.25 mg/kg body weight) and MPA on the E_1 (1.0 μg, s.c., twice daily)-stimulated growth of DMBA-induced mammary carcinoma in ovariectomized rats. The change in tumor size is expressed as % of initial tumor size. The data are expressed as means ± SEM [125].

simultaneous administration of the pure antiandrogen Flutamide [133]. Taken together, the present data indicate the involvement of the androgen receptor in the chemopreventive action of DHEA. Since antiestrogens and DHEA exert chemopreventive effects on breast cancer via different mechanisms, it is reasonable to expect that the combination of EM-800 and DHEA exerts more potent inhibitory effects than each compound used alone on the development of DMBA-induced rat mammary carcinoma as well illustrated by the present data.

5.3. Inhibition of estrone-stimulated growth of DMBA-induced mammary carcinoma in the rat—combination with MPA

In the present study, in addition to investigating the effect of the pure antiestrogen EM-800 on the E_1-stimulated growth of DMBA-induced mammary carcinoma, we have studied the antitumoral activity of the combination of EM-800 with the androgenic medroxyprogesterone acetate (MPA) at submaximal doses.

After 65 days of treatment with estrone, the average tumor area reached 225 ± 40.9% of initial tumor size, while in the OVX group (in the absence of estrogens), the tumors continuously regressed to only 9.6 ± 3.9% of initial tumor size ($p < 0.01$). Treatment with MPA reversed by 84.1% the stimulatory effect of estrone and decreased tumor area to 44 ± 9.6% of initial size ($p < 0.01$). On the other hand, treatment with EM-800 reversed by 82.9% the stimulatory effect of estrone at the low dose of 0.25 mg per kg body weight while the stimulatory effect of estrone was completely abolished at the high dose of EM-800, a value of 10.4 ± 3.5% of initial size being measured ($p < 0.01$). It is of interest to note that combination of the low dose EM-800 (0.25 mg per kg body weight) and MPA further inhib-

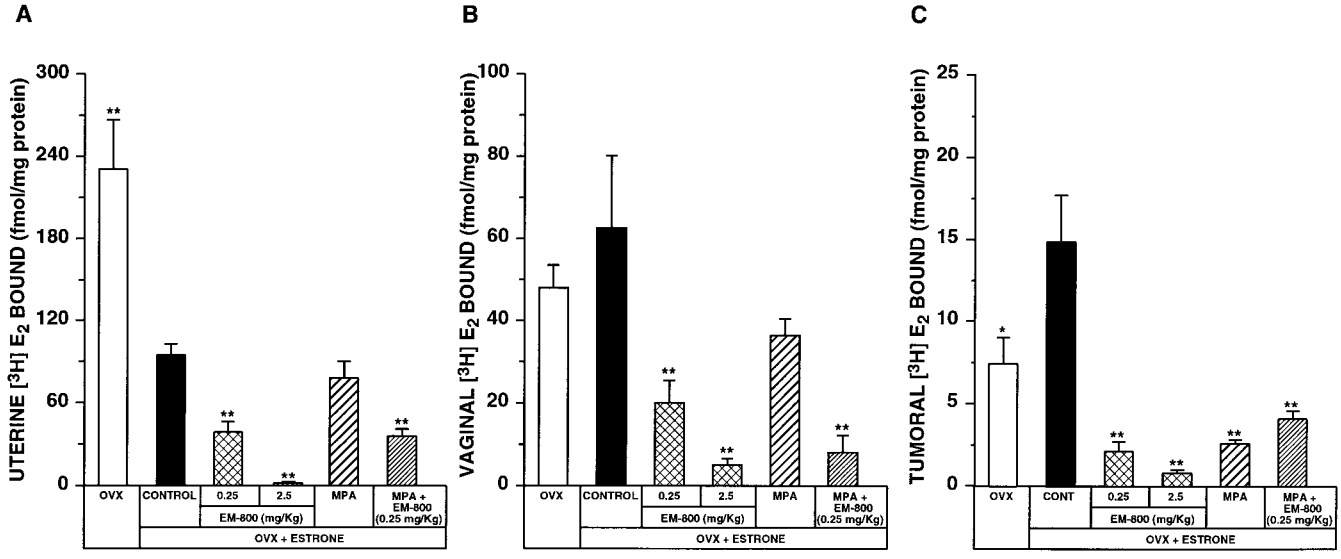

Fig. 15. Effect of 65-day-treatment with the antiestrogen EM-800 at the doses of 0.25 and 2.5 mg per kg body weight (orally, once daily) or medroxyprogesterone acetate (MPA, 1 mg s.c., twice daily) or the combination of EM-800 (0.25 mg per kg body weight) and MPA on uterine (A), vaginal (B), and tumoral (C) estrogen receptor levels in ovariectomized rats supplemented with E_1 (1 μg, s.c., twice daily). $^{*}P < 0.05$; $^{**}P < 0.01$ vs OVX + E_1 control [125].

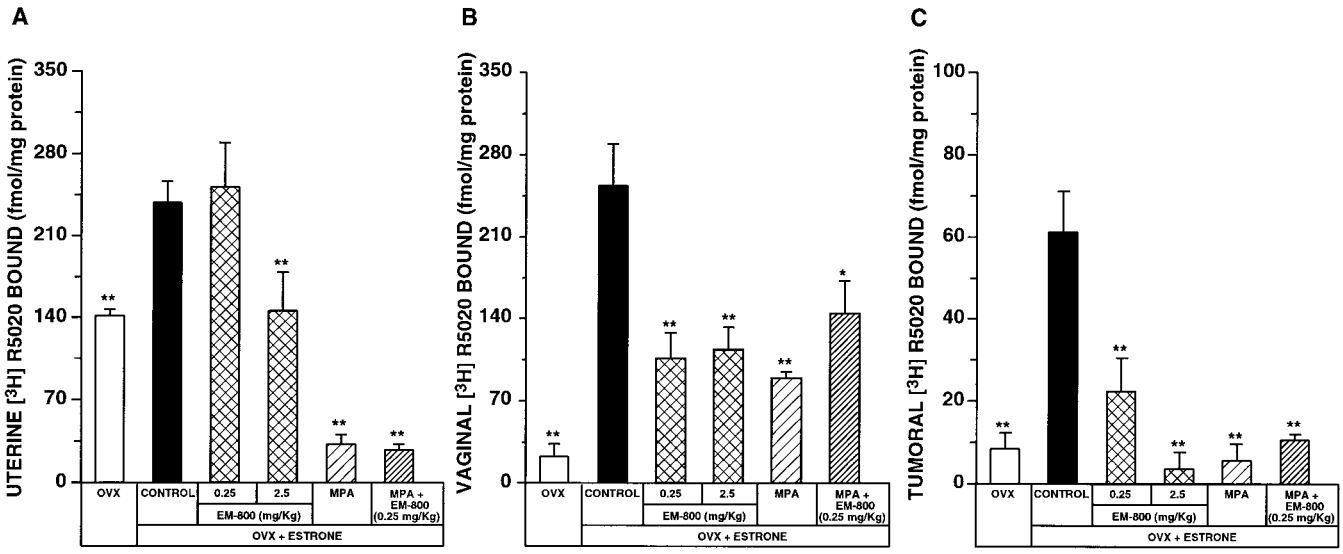

Fig. 16. Effect of 65-day-treatment with the antiestrogen EM-800 at the doses of 0.25 and 2.5 mg per kg body weight (orally, once daily) or medroxyprogesterone acetate (MPA, 1 mg s.c., twice daily) or the combination of EM-800 (0.25 mg per kg body weight) and MPA on uterine (A), vaginal (B) and tumoral (C) progesterone receptor levels in ovariectomized rats supplemented with E_1 (1 μg, s.c., twice daily). $^{*}P < 0.05$; $^{**}P < 0.01$ vs OVX + E_1 control [125].

ited E_1-stimulated tumor growth to 16.2 ± 5.7%, a more potent inhibitory effect than that achieved with each compound used alone. ($p < 0.05$ versus MPA alone, but NS versus low EM-800 dose alone) (Fig. 14).

5.4. Effects of EM-800 on uterine, vaginal and tumoral steroid receptor levels

Supplementation of OVX rats with E_1 decreased uterine [^{3}H]E_2 binding from 230 ± 35.7 to 94.9 ± 8.1 fmol/mg protein ($p < 0.01$) (Fig. 15A) while no significant effect was observed on vaginal ER levels (Fig. 15B). On the other hand, increased ER levels from 7.5 ± 1.6 to 14.8 ± 2.9 fmol/mg protein ($p < 0.05$) were measured in the DMBA-induced mammary tumors (Fig. 15C). In addition, E_1-supplementation increased uterine PR levels from 142 ± 5.4 to 238 ± 18.2 fmol/mg protein ($p < 0.01$) (Fig. 16A) and vaginal PR from 23.0 ± 10.9 to 253 ± 35.3 fmol/mg protein ($p < 0.01$) (Fig. 16B) as well as tumoral PR levels from 8.5 ± 3.8 to 61.0 ± 10.0 fmol/mg protein ($p < 0.01$) (Fig. 16C). Treatment with EM-800 significantly inhibited uterine, vaginal, and tumoral ER levels in a dose-related fashion (Fig. 15). In agreement with the decrease of ER concentrations, uterine, vaginal, and tumoral PR contents were also decreased by the same treatment with EM-800 (Fig. 16). Treatment with MPA, at the dose used, decreased ER levels only in the tumoral tissue while it had no significant effect in the uterus or in the vagina (Fig. 15). On the other hand, MPA significantly reduced PR levels in all the three tissues examined (Fig. 16). The combination of the low dose EM-800 (0.25 mg/kg body weight) and MPA inhibited ER (Fig. 15) and PR (Fig. 16) levels in the above-mentioned three tissues.

Since E_1 is the predominant circulating estrogen precursor in postmenopausal women, the use of E_1 -supplementation in OVX rats bearing DMBA-induced mammary tumors provides a model which mimics the conditions of postmenopausal breast cancer [134]. Using this model, the present data show that the novel antiestrogen EM-800 and MPA both exert potent inhibitory effects on the E_1-stimulated growth of DMBA-induced mammary carcinomas. Most importantly, the combination of the two compounds at submaximal doses caused a greater inhibition than achieved by each compound used alone, thus reaching 96.9% inhibition of the E_1-stimulated growth of mammary carcinoma induced by DMBA. Moreover, EM-800 alone, at the higher dose of 2.5 mg/kg body weight, completely abolished the stimulatory effect of E_1 on the growth of these tumors, thus demonstrating the particularly high efficacy of this novel antiestrogen.

The present data, in addition, show that EM-800 decreases ER levels in the three estrogen-sensitive tissues studied, namely the uterus, vagina, and DMBA-induced tumors. In order to exclude the possibility that the down-regulation observed in the present study is due to the masking effect of EM-800 on the availability of ER sites to radiolabelled E_2, we have measured ER content using the estrogen receptor enzyme immunoassay (ER-EIA, Abbott kit). The results obtained with the ER-EIA were parallel to

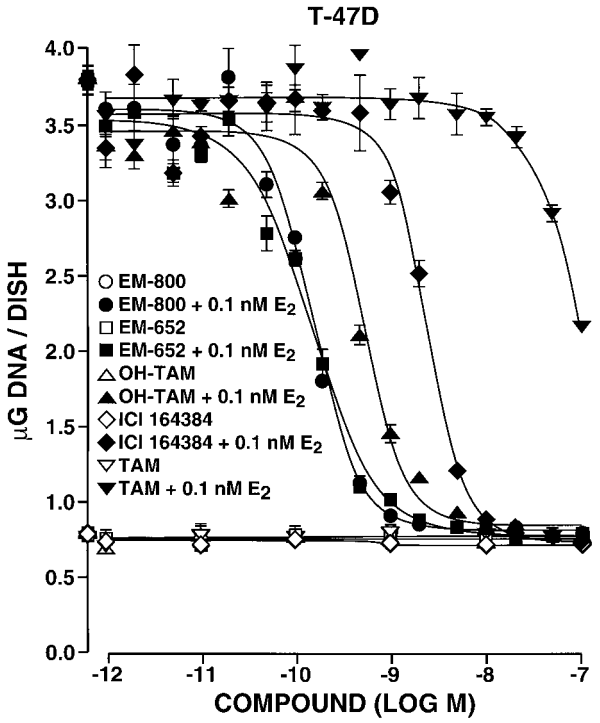

Fig. 17. Effect of increasing concentrations of EM-652, EM-800, ICI 164384, 4-OH-trans-Tamoxifen (OH-TAM) or Tamoxifen (TAM) on basal and E_2-induced cell proliferation in T-47D human breast cancer cells. Three days after plating, at an initial density of 7.5×10^3/2-cm^2 well, cells were exposed for 10 days to the indicated concentrations of compounds in the presence or absence of 0.1 nM E_2. Media were changed at 2- or 3-day intervals. The K_i values of EM-800, EM-652, OH-TAM, ICI 164384 and TAM for their effect on E_2-induced cell proliferation were calculated at 0.015, 0.015, 0.053, 0.243 and ~10 nM, respectively. Data are expressed as the means ± SEM of triplicate dishes when the SEM overlaps with the symbol used. Only the symbol is illustrated [70].

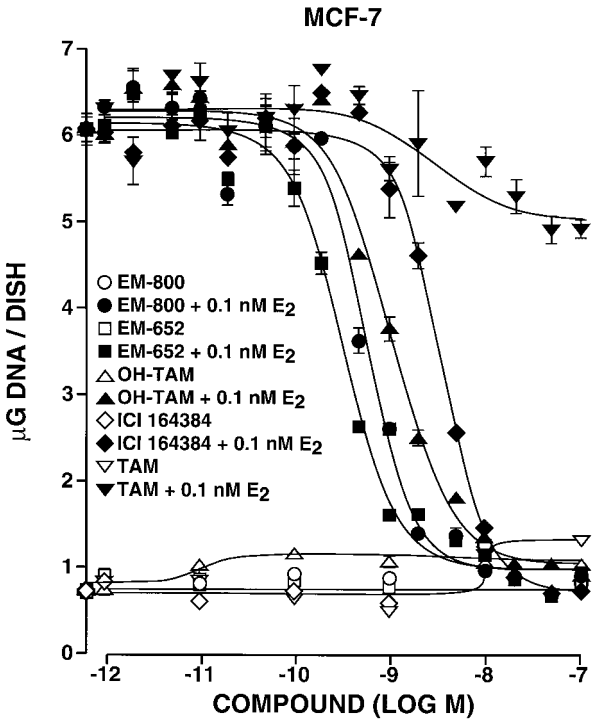

Fig. 18. Effect of increasing concentrations of EM-652, EM-800, ICI-164384, 4-OH-trans-Tamoxifen (OH-TAM) or Tamoxifen (TAM) on basal and E_2-induced cell proliferation in MCF-7 human breast cancer cells. Three days after plating, at an initial density of 1×10^4/2-cm^2 well, cells were exposed for 9 days to the indicated concentrations of compounds in the presence or absence of 0.1 nM E_2. Media were changed at 2- or 3-day intervals. Data are expressed as described in the legend of Fig. 17. The K_i values of EM-800, EM-652, OH-TAM and ICI 164384 for their effect on E_2-induced cell proliferation were calculated at 0.033, 0.018, 0.060 and 0.197 nM, respectively [70].

those described above for the radioligand assay (data not shown), thus confirming the observation that EM-800 markedly decreases ER protein as well as binding levels in the tissues examined.

6. Inhibition of the growth of human breast cancer cell lines in vitro and in vivo in nude mice

6.1. Inhibition of the growth of human breast cancer ZR-75-1, MCF-7 and T-47D cells in vitro

The present study describes the effects of the novel non-steroidal antiestrogen EM-652 and related compounds on basal and E_2-induced cell proliferation in three well characterized estrogen receptor-positive human breast cancer cell lines. The present results show that EM-652 and its precursor EM-800 are the most potent known antiestrogens in vitro in human breast cancer cells and are, most importantly, devoid of any intrinsic estrogenic activity.

6.1.1. Comparison of the effects of EM-652 and EM-800 with those of ICI 164384, OH-TAM and TAM on basal and E_2-induced cell proliferation in T-47D, ZR-75-1 and MCF-7 human breast cancer cell lines

Since EM-800 is rapidly metabolized into the active compound EM-652 in intact cells, we compared the effect of increasing concentrations of the non-steroidal antiestrogens EM-652 and EM-800 with those of OH-TAM and TAM and of the steroidal antiestrogen ICI 164384 on basal and E_2-induced cell proliferation in T-47D, ZR-75-1 and MCF-7 cells [70]. As illustrated in Fig. 17, a 10-day exposure to 0.1 nM E_2 increased the proliferation of T-47D cells by 4.77-fold. This E_2-induced stimulation of cell proliferation was competitively blocked by simultaneous incubation with EM-800, EM-652, OH-TAM, ICI 164384 and TAM at respective IC_{50} values of 0.148, 0.146, 0.522, 2.41 and ~100 nM. It can also be seen in Fig. 17 that none of

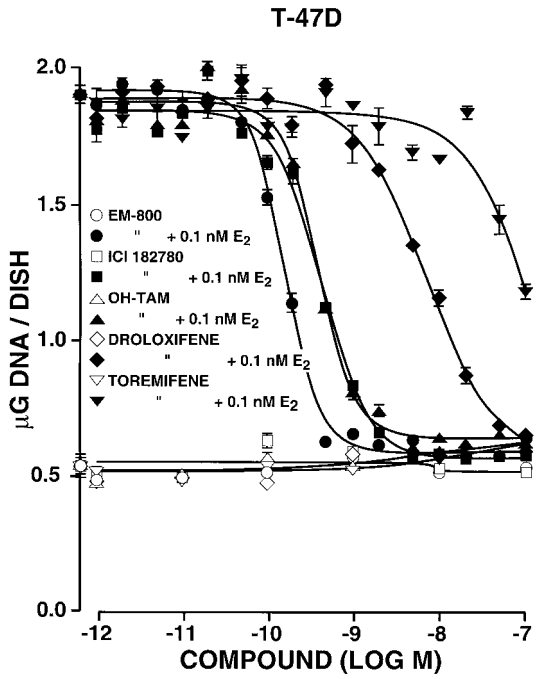

Fig. 19. Effect of increasing concentrations of EM-800, ICI-182780 or 4-OH-*trans*-Tamoxifen (OH-TAM) Droloxifene or Toremifene on basal and E_2-induced cell proliferation in T-47D human breast cancer cells. Three days after plating cells were exposed for 9 days to the indicated concentrations of compounds in the presence or absence of 0.1 nM E_2. Media were changed at 2- or 3-day intervals. Data are expressed as described in the legend of Fig. 17. The K_i values of EM-800, OH-TAM, ICI-182780, Droloxifene or Toremifene for their effect on E_2-induced cell proliferation were calculated at 0.016, 0.040, 0.044, 0.735 and >10 nM, respectively [70].

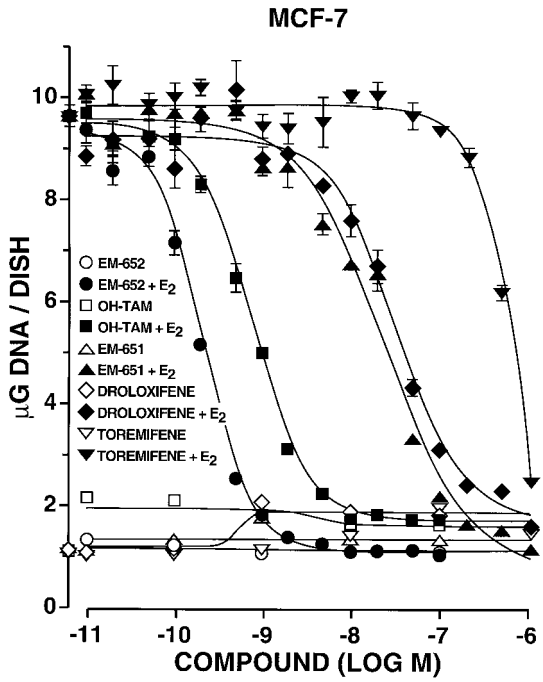

Fig. 20. Effect of increasing concentrations of EM-652, 4-OH-trans-Tamoxifen (OH-TAM), EM-651, Droloxifene or Toremifene on basal and E_2-induced cell proliferation in MCF-7 human breast cancer cells. Three days after plating, at an initial density of 1×10^4/2-cm^2 well, cells were exposed for 8 days to the indicated concentrations of compounds in the presence or absence of 0.1 nM E_2. Media were changed at 2- or 3-day intervals. Data are expressed as described in the legend of Fig. 17. The K_i values of EM-652, OH-TAM, EM-651, Droloxifene, and Toremifene for their effect on E_2-induced cell proliferation were calculated at 0.011, 0.041, 1.29, 1.73, and >28 nM [70].

these compounds did affect basal T47-D cell proliferation when incubated alone.

After a 9-day incubation of ZR-75-1 cells with increasing concentrations of EM-800, EM-652, OH-TAM, ICI 164384 or TAM, the 2.01-fold increase in cell proliferation induced by 0.1 nM E_2 was competitively reversed at respective IC_{50} values of 0.475, 0.750, 0.646, 4.88 and ~100 nM (data not shown [70]). Furthermore, basal cell proliferation was not significantly affected after a 9-day incubation with increasing concentrations of EM-652 or EM-800 in the absence of E_2. In the absence of estrogens, OH-TAM, on the other hand, led to a 10% ($p < 0.05$) stimulation of cell proliferation.

The 8.43-fold increase in MCF-7 cell proliferation induced by a 9-day incubation with 0.1 nM E_2 was competitively blocked by a simultaneous exposure to EM-800, EM-652, OH-TAM or ICI 164384 at respective IC_{50} values of 0.582, 0.321, 1.06 and 3.49 nM (Fig. 18). However, such an incubation with 50 and 100 nM TAM was only able to reverse by ~20% the E_2-induced MCF-7 cell proliferation. It can also be seen in Fig. 18 that after a 9-day incubation with increasing concentrations of EM-652 or EM-800 in the absence of E_2, the basal proliferation of MCF-7 cells was not affected significantly. OH-TAM, on the other hand, at the concentrations of 0.1–10 nM caused 35% to 55% ($p < 0.01$) stimulation of cell proliferation in the absence of E_2.

6.1.2. Comparison of the effect of EM-800 with those of Droloxifene, OH-Toremifene, Toremifene and ICI-182780 on basal and E_2-induced human breast cancer cell proliferation in T47-D and MCF-7 human breast cancer cells

Since Droloxifene, Toremifene and ICI-182780 are being developed for the treatment of breast cancer [63,135,136], it was of interest to compare the effect of these three compounds on breast cancer cell proliferation to that of EM-800. As illustrated in Fig. 19, a 9-day exposure to 0.1 nM E_2 increased by 3.8-fold the proliferation of T-47D cells. This E_2-induced stimulation of cell proliferation was competitively blocked by simultaneous incubation with EM-800, OH-TAM, ICI-182780, Droloxifene or Toremifene at respective

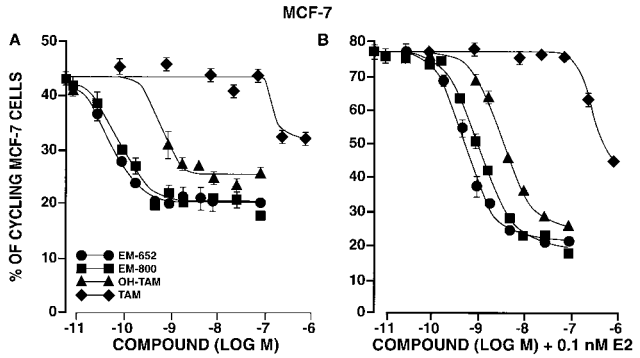

Fig. 21. Effect of increasing concentrations of EM-652, EM-800, OH-Tamoxifen (OH-TAM) or Tamoxifen (TAM) on the proportion of cycling MCF-7 cells after exposure to BrdUrd. Three days after plating at an initial density of 0.85×10^5/10-cm^2 well, cells were pretreated for 3 days with the indicated concentrations of compounds in the presence (Panel B) or absence (Panel A) of 0.1 nM E_2 before changing to fresh medium containing the same compounds and 10 μM BrdUrd. Cells were then harvested after two days, fixed and stained with the dye Hoechst 33358. The percentage of BrdUrd-positive cells was calculated as described in Materials and methods. Data obtained with control medium alone in the presence or absence of 0.1 nM E_2 are indicated on the *y*-axis. The data are expressed as the means ± SEM of triplicate dishes. When the SEM overlaps with the symbol used, only the symbol is illustrated [70].

IC_{50} values of 0.158, 0.400, 0.434, 7.30 and >100 nM (Fig. 19).

After 8 days of treatment of MCF-7 cells in the absence of E_2, OH-TAM, Droloxifene, and Toremifene all led to a 75–100% increase in cell proliferation which was dose-dependent: the stimulation by OH-TAM was observed at concentrations as low as 0.01 nM, the lowest concentration used, while Droloxifene gave the first significant effect at 1 nM and Toremifene stimulated basal MCF-7 cell proliferation at 100 nM (Fig. 20). In the same experiment, EM-652, OH-TAM, Droloxifene and Toremifene inhibited the proliferative action of E_2 at respective IC_{50} values of 0.193, 0.731, 30.58, and >500 nM. In fact, it is of interest to note that after a 9-day incubation with increasing concentrations of EM-800, OH-Toremifene or Toremifene in T-47D cells, the 2.47-fold increase in cell proliferation induced by 0.1 nM E_2 was reversed at respective IC_{50} values of 0.112, 0.430, and 179 nM [70, data not shown].

6.1.3. Comparison of the effect of EM-652, EM-800 and TAM on the proportion of cycling MCF-7 cells

To assess the percentage of MCF-7 cells that progressed through the S-phase of the cycle during incubation with EM-652, EM-800 or TAM in the presence or absence of E_2, the continuous BrdUrd exposure technique was used. As measured after a 48-h exposure to BrdUrd, 72-h pretreatment with 1 nM EM-652, EM-800 or OH-TAM alone decreased the percentage of BrdUrd-positive cells from 43.6% to 20.2%, 21.5% and 30.9%, respectively ($p < 0.01$) (Fig. 21A). On the other hand, incubation with 0.1 nM E_2 increased the percentage of BrdUrd-positive cells to 77.9% ($p < 0.01$). Addition of increasing concentrations of EM-652, EM-800 or OH-TAM completely blocked the stimulatory effect of E_2 on this parameter and caused a further decrease below the control value to reach levels similar to those obtained with these compounds in the absence of E_2 (Fig. 21B). The inhibitory effect of EM-652, EM-800 and OH-TAM on the percentage of BrdUrd-positive cells was observed at respective IC_{50} values of 0.60, 1.26 and 3.8 nM. It can also be seen in Fig. 21 that treatment with TAM was approximately 1000-fold less effective to decrease the proportion of cycling MCF-7 cells.

The present data show that the novel non-steroidal compound EM-800 and its metabolite EM-652 exert the most potent antagonistic effects of the compounds tested on E_2 -induced proliferation in T-47D, ZR-75-1 and MCF-7 human breast cancer cells in vitro. Furthermore, the present data indicate that these two antiestrogens are devoid of intrinsic estrogenic activity as evidenced by the absence of any stimulatory effect on basal cell proliferation in three estrogen-sensitive human breast cancer cell lines.

The present study also shows that EM-652 and EM-800 decrease the proportion of MCF-7 cells which advanced through the S phase, and completely block the stimulatory effect of E_2 on this parameter. In fact, EM-652 and EM-800 were at least 1000-fold more effective than TAM in reducing the proportion of BrdUrd-positive cells in the presence or absence of E_2 while EM-652 was 6-fold more potent than OH-TAM in the presence of E_2.

Most importantly, EM-800 and EM-652 have no estrogenic activity in the three breast cancer cell lines studied while OH-TAM and Droloxifene cause a significant stimulation of ZR-75-1 and of MCF-7 human breast cancer cell proliferation. The estrogenic activity of TAM is illustrated by studies demonstrating tumor response to withdrawal of TAM at time of treatment failure [31,32]. Such data suggest that progression of the disease observed in patients under TAM is related to the stimulation of the cancer by the estrogenic activity of TAM. These clinical data are well supported by the stimulation induced by TAM of breast cancer cells cultured directly from patients [137 and refs therein]. The observations of a stimulatory effect of TAM on breast cancer cell growth are in agreement with the stimulatory effect of TAM or OH-TAM observed on the growth of breast cancer cells demonstrated repeatedly in vitro [28,68 and refs therein, 137] as well as in vivo [138].

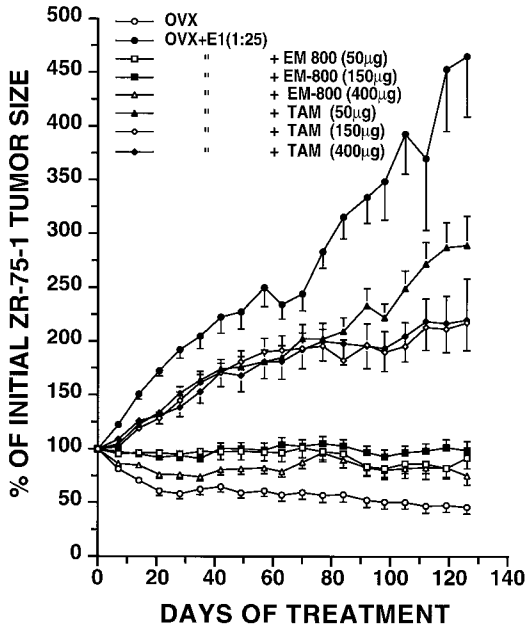

Fig. 22. Time-course of the effect of treatment with the pure antiestrogen EM-800 or Tamoxifen at the daily oral dose of 50, 150 or 400 μg for 4 months on the average size of ZR-75-1 human breast cancer xenografts in ovariectomized nude mice supplemented with an implant of estrone. The size of tumors at start of treatment was 31.1 $\pm$ 0.8 mm^2. Ovariectomized mice receiving the vehicle alone were used as additional controls. Results are expressed as percentage of pretreatment values (means $\pm$ SEM of 28 to 37 tumors per group) [71].

6.2. Comparison of the effect of EM-800 and Tamoxifen on the growth of human breast cancer xenografts in nude mice

Tamoxifen has shown important benefits in breast cancer and has become the standard therapy at all stages of the disease. Although 30–50% of the patients with advanced breast cancer show a positive response to Tamoxifen, the duration of response is usually limited to 12–18 months with the development of resistance to further treatment with this antiestrogen [2,3,139]. As mentioned above and demonstrated in a series of studies with human breast cancer cell lines in vitro and in vivo [28,29,37,39,140,141] and supported by clinical observations [27,31,32,142–144], it seems reasonable to suggest that the loss of positive response to Tamoxifen treatment in breast cancer patients could be, at least in part, due to the intrinsic estrogenic activity of the compound. This explanation is supported by the finding that human breast cancer cell lines showing resistance to Tamoxifen retain their sensitivity to specific antiestrogens in vitro [141,145–147] as well as in vivo in nude mice [140,148,149].

Since human breast carcinoma xenografts in nude mice are the closest available model of human breast cancer, we have compared the effect of EM-800 and Tamoxifen alone and in combination on the growth of ZR-75-1 breast cancer xenografts in nude mice.

While estrone caused a 365% increase in ZR-75 tumor size during the 4-month treatment period, administration of the oral daily 50, 150 or 400 μg dose of the antiestrogen EM-800 completely prevented tumor growth (Fig. 22). In fact, at the 400 μg dose, average tumor size was reduced by 25% ($p < 0.001$) at 4 months compared to the initial size at start of treatment. When the same doses of Tamoxifen were administered, it can be seen in the same figure that average tumor sizes were measured at 189, 117 and 120% above pretreatment values at the 50, 150 and 400 μg doses, respectively ($p < 0.0001$ for all doses).

When EM-800 at the daily oral dose of 150 μg was combined with the same dose of Tamoxifen, average tumor size decreased from 117% for Tamoxifen alone to 12% for Tamoxifen + EM-800 ($p < 0.001$) (Fig. 23). When the higher dose of Tamoxifen was used, namely 400 μg daily, the addition of 150 μg of EM-800 decreased tumor size from 120% above average initial size for Tamoxifen alone to 38% for Tamoxifen + EM-800 ($p < 0.01$, Fig. 23B). In the presence of EM-800, average tumor size was not significantly different from the pretreatment values.

While the above-described results were obtained in OVX animals supplemented with estrone, it can be seen in Fig. 24 that the administration of Tamoxifen alone in ovariectomized animals not supplemented with estrone stimulated tumor growth. In fact, after 4 months of treatment with Tamoxifen alone, average tumor size was increased to 161 $\pm$ 20%, ($p < 0.001$) above pretreatment values, while administration of EM-800 led to values superimposable to those obtained in the absence of estrogen in ovariectomized animals receiving the vehicle alone, namely 55% below initial tumor size ($p < 0.0001$, Fig. 24). Such data are a direct demonstration of the intrinsic stimulatory activity of Tamoxifen on human breast cancer growth.

The present data clearly show, under in vivo conditions in nude mice, the stimulatory effect of Tamoxifen on the growth of human breast cancer xenografts while the novel antiestrogen EM-800 has no stimulatory effect. In fact, 73% of tumors progressed when Tamoxifen alone was administered to ovariectomized animals while no tumor progressed with EM-800. Moreover, in ovariectomized animals supplemented with estrone, EM-800 (150 μg daily) could completely neutralize the increase in average tumor size observed with Tamoxifen at both the 150 μg (Fig. 22A) and 400 μg (Fig. 22B) doses. The present demonstration of a stimulatory effect of Tamoxifen on human breast cancer growth is in agreement with previous data obtained in human breast cancer cell lines in vitro [28,37,39,141] as well as in vivo in nude mice [29,140]. These experimental data are also in agreement

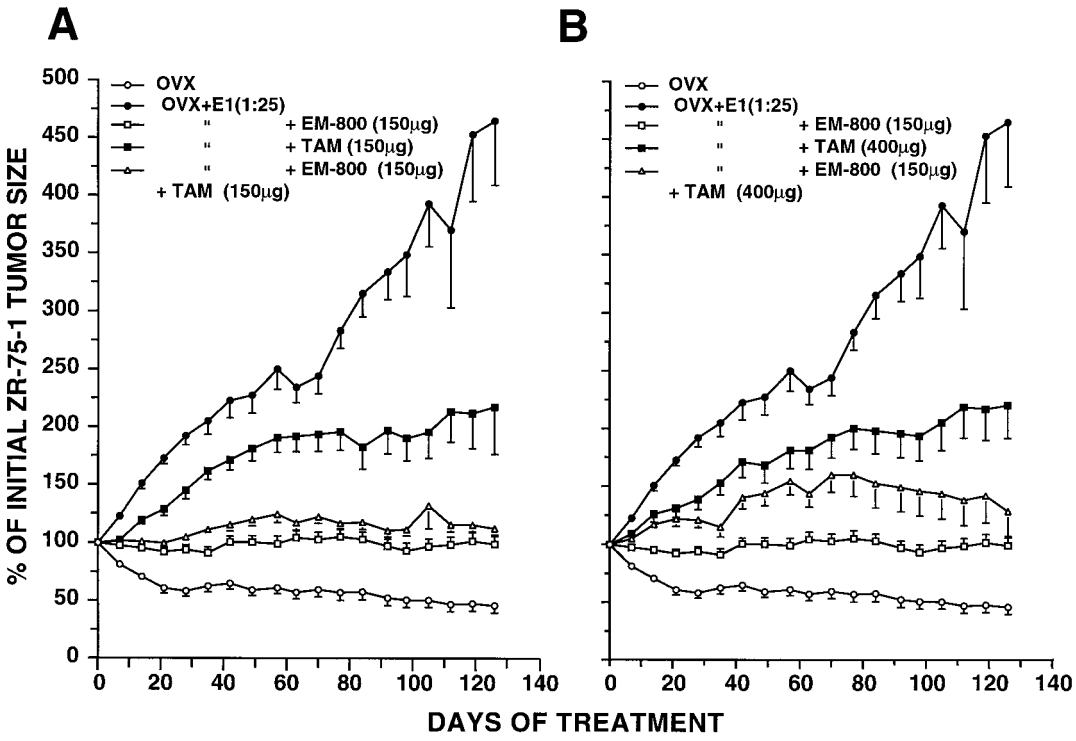

Fig. 23. Time-course of the effect of (A) daily oral doses of 150 μg of EM-800, 150 μg of Tamoxifen or the combination of both drugs; or (B) daily oral doses of 150 μg of EM-800, 400 μg of Tamoxifen or the combination of both drugs for 4 months on the average size of ZR-75-1 human breast cancer xenografts in ovariectomized nude mice supplemented with an implant of estrone. Ovariectomized nude mice receiving the vehicle alone or supplemented with estrone implants are added as controls. The size of tumors at start of treatment was 31.1 ± 0.8 mm^2. Results are expressed as percentage of pretreatment values (means $\pm$ SEM of 25–37 tumors per group) [71].

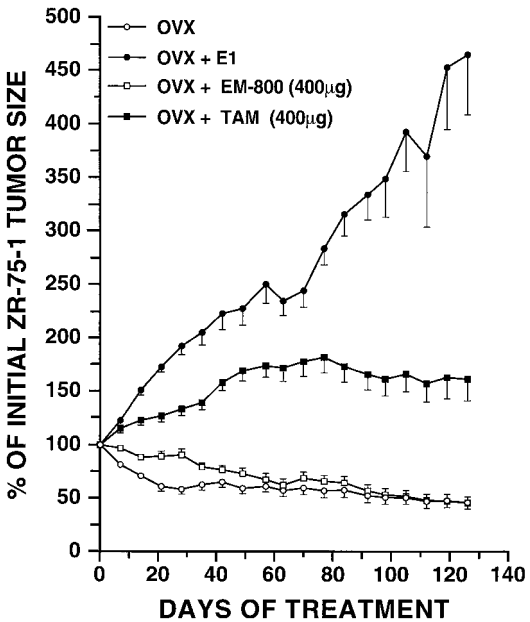

Fig. 24. Time-course of the effect of the daily oral dose of 400 μg of EM-800 or Tamoxifen on the average size of ZR-75-1 human breast cancer xenografts in ovariectomized nude mice. Ovariectomized nude mice receiving the vehicle alone or supplemented with estrone implants are added as controls. The size of tumors at start of treatment was 31.1 ± 0.8 mm^2. Results are expressed as percentage of pretreatment values (means $\pm$ SEM of 28–37 tumors per group) [71].

with clinical observations suggesting the stimulatory effect of Tamoxifen on breast cancer in women [27,31,32,142–144]. Particularly convincing evidence of the estrogenic activity of Tamoxifen is also provided by the finding that human breast cancer cell lines showing resistance to Tamoxifen retain their sensitivity to specific or pure antiestrogens in vitro [141,145–147] as well as in vivo in nude mice [140,148,149].

Although adjuvant treatment with Tamoxifen delays breast cancer recurrence and improves survival in early breast cancer and induces remission in patients with advanced disease, its benefits are ultimately limited by the development of Tamoxifen resistance [150]. Similarly, in the in vivo model using nude mice, Tamoxifen inhibited MCF-7 tumor growth for 4–6 months but tumor growth then continued despite Tamoxifen treatment [149,151]. In analogy with the present data, Gottardis et al. [29] have observed the acquired ability of Tamoxifen to stimulate rather than to inhibit tumor growth. Since, as mentioned above, pure antiestrogens can inhibit the stimulatory effect of Tamoxifen [71,140,148,150] (Fig. 23), such data suggest that the stimulatory effect of Tamoxifen upon long-term treatment is due to the intrinsic estrogenic activity of the compound or its metabolites [152].

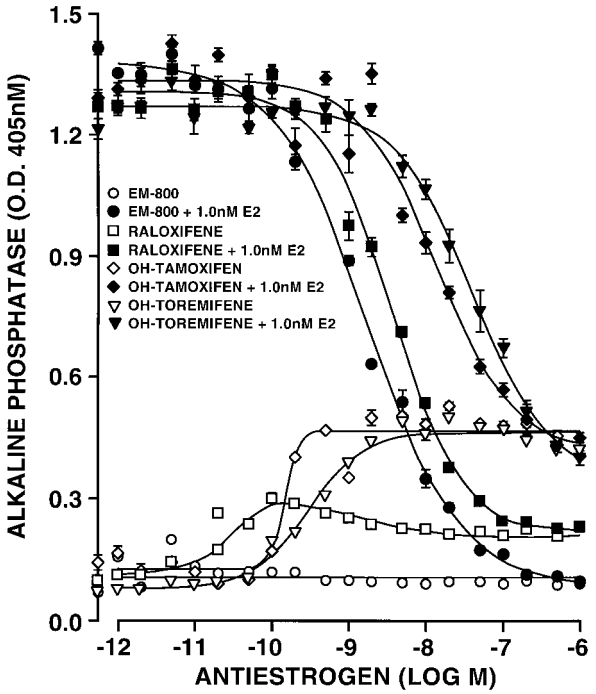

Fig. 25. Effect of increasing concentrations of EM-800, (Z)-4-OH-Tamoxifen, (Z)-4-OH-Toremifene and Raloxifene on alkaline phosphatase activity in human Ishikawa cells. Alkaline phosphatase activity was measured after a 5-day exposure to increasing concentrations of the indicated compounds in the presence or absence of 1.0 nM E_2. The data are expressed as the means ± SEM of four wells. When the SEM overlaps with the symbol used, only the symbol is shown [57].

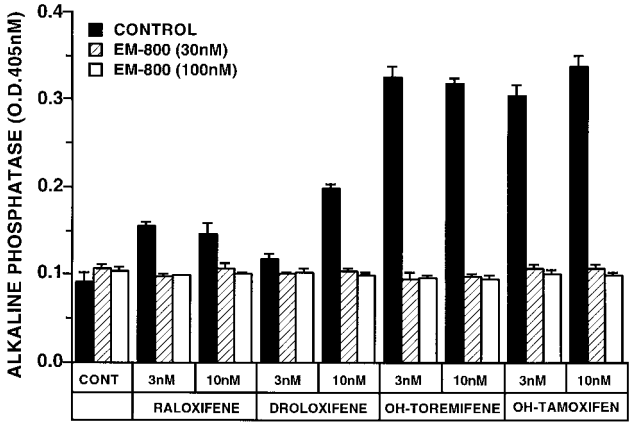

Fig. 26. Blockade of the stimulatory effect of (Z)-4-OH-Tamoxifen, (Z)-4-OH-Toremifene, Droloxifene and Raloxifene on alkaline phosphatase activity by the antiestrogen EM-800 in human Ishikawa carcinoma cells. Alkaline phosphatase activity was measured after a 5-day exposure to 3 or 10 nM of the indicated compounds in the presence or absence of 30 or 100 nM EM-800. The data are expressed as the means ± SD of eight wells with the exception of the control groups were data are obtained from 16 wells [57].

Treatment of nude mice bearing MCF-7 xenografts with 10 mg ICI 182780 once a week led to a transient decrease of tumor size followed by a plateau of no change for about 200 days followed by progression [140]. In mice treated with ICI 182780, regrowth of tumors or resistance to ICI 182780 occurred in most tumors [140].

The stimulatory effect of Tamoxifen or OH-Tamoxifen on human breast cancer cell growth has been reported previously by many laboratories under in vitro [28,34–37,68,137,153–157] as well as in vivo [29] conditions. Such intrinsic estrogenic activity of Tamoxifen probably limits its success in the treatment of breast cancer in women [23]. In addition to the data mentioned earlier, the estrogenic action of Tamoxifen in breast cancer in women is supported clinically by the tumor flare observed at start of therapy [158–160]. This early stimulatory effect of Tamoxifen is analogous to the present data showing a stimulatory effect of the same drug on the growth of ZR-75-1 xenografts. The withdrawal response observed following arrest of Tamoxifen in patients who progress under Tamoxifen therapy [31,32] can also be explained as a result of withdrawing the estrogenic activity of Tamoxifen.

7. Pure antiestrogenic activity of EM-652 and EM-800 in human endometrial adenocarcinoma Ishikawa cells

Since data suggest that continuous long-term Tamoxifen therapy is preferable to its usual short-term use [161] and studies are already in progress on the long-term administration of Tamoxifen to prevent breast cancer [54,61], it becomes important to make available a pure antiestrogen which, due to its lack of estrogenic activity, should theoretically be more efficient than Tamoxifen to treat breast cancer while simultaneously eliminating the excess risk of developing uterine carcinoma during its long-term use. The present study compares the effect of EM-800 or its active metabolite EM-652 with those of OH-Tamoxifen, OH-Toremifene, Droloxifene, Raloxifene and ICI-182780 [63,135,136,162,163] on estrogen-sensitive alkaline phosphatase (AP) activity in human endometrial carcinoma Ishikawa cells. AP activity is well known to be stimulated by estrogens, while the other steroids, namely androgens, progestins, mineralocorticoids or glucocorticoids, have no effect on this parameter [164].

As illustrated in Fig. 25 exposure to 0.1, 1, 10 and 100 nM Raloxifene increased by 3.0-, 2.5-, 2.3- and 2.1-fold, respectively, alkaline phosphatase activity. It can also be seen in this figure that the marked stimulatory effect exerted by 1 nM E_2 was competitively, but not completely, reversed by (Z)-4-OH-Tamoxifen, (Z)-4-OH-Toremifene, and Raloxifene, their partial inhibitory action being exerted at an IC_{50} values 13.5 ± 3.8 nM, 41.0 ± 7.2 nM and 3.74 ± 0.43 nM, respectively, while the E_2-induced alkaline phosphatase activity was completely blocked by a simultaneous

exposure to EM-800 at an IC_{50} value of 1.73 ± 0.19 nM.

Direct comparison of the estrogen-like activity of these antiestrogens can best be made in Fig. 26. Incubation with 3 nM (Z)-4-OH-Tamoxifen, (Z)-4-OH-Toremifene, Droloxifene, or Raloxifene increased the value of alkaline phosphatase activity by 3.3-, 3.6-, 1.3- and 1.7-fold, respectively, while exposure to 10 nM of these compounds increased the value of the same parameter by 3.3-, 3.5-, 2.2-, and 1.6-fold, respectively (Fig. 26). The complete blockade of the stimulatory effect of all these antiestrogens on AP activity by simultaneous exposure to EM-800 well supports the suggestion that the stimulatory effect of (Z)-4-OH-Tamoxifen, (Z)-4-OH-Toremifene, Droloxifene and Raloxifene on this parameter is mediated through the estrogen receptor (Fig. 26).

The present data clearly demonstrate that the novel nonsteroidal antiestrogen EM-800 and its active metabolite EM-652 exert pure antagonistic effects while being the most potent of the compounds tested on E_2-induced alkaline phosphatase activity in human endometrial adenocarcinoma Ishikawa cells. In contrast to EM-800 and EM-652, (Z)-4-OH-Tamoxifen, (Z)-4-OH-Toremifene, Droloxifene and Raloxifene exert a stimulatory effect on this estrogen-sensitive parameter, an effect which can be completely blocked by simultaneous exposure to the antiestrogen EM-800, thus well supporting the suggestion that the stimulatory effect of these antiestrogens is mediated through activation of the estrogen receptor.

The appearance of uterine carcinoma in women treated with Tamoxifen [165,166 and refs. therein], is not surprising since Tamoxifen has been shown to stimulate the growth of two human endometrial tumors implanted in nude mice [29,167,168] as well as in vitro [169–171]. Furthermore, OH-Tamoxifen has been shown to be potent and sometimes even more potent than E_2 itself to stimulate progesterone receptors in the human Ishikawa endometrial cell line [171]. It should be added that the relationship between estrogens and endometrial carcinoma is well known [172–174]. As further support to the data obtained in human endometrial carcinoma, the potent stimulatory effect of Tamoxifen on estrogen-sensitive parameters in the normal uterus is also well known in the mouse, rat and hamster [19,175,176]. It thus appears that the estrogenic activity of Tamoxifen in the uterus is common to all estrogen-sensitive parameters and species so far studied. It is thus not surprising that the two other Tamoxifen-related compounds namely, (Z)4-OH-Toremifene and Droloxifene also possess an estrogenic activity in human Ishikawa cells. Similar stimulatory effects on endometrial alkaline phosphatase are also found with another analog of Tamoxifen, namely Idoxifene, as well as with LY 353381, an analog of Raloxifene (data not shown).

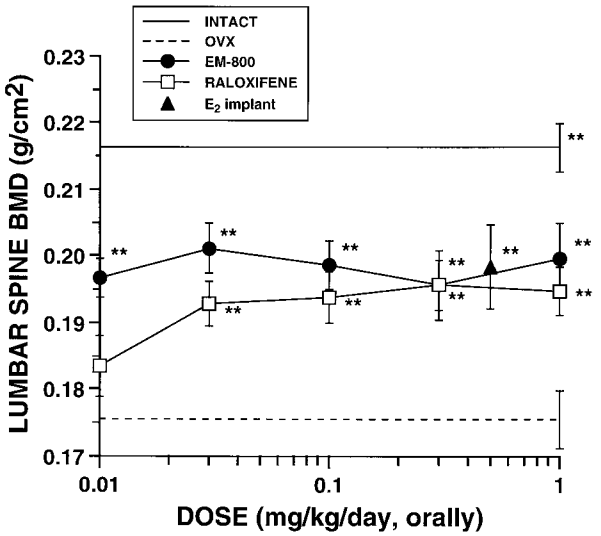

Fig. 27. Effect of 35-week treatment with increasing daily oral doses of EM-800 or Raloxifene on lumbar spine bone mineral density (BMD) in ovariectomized rats. Comparison is made with intact rats and ovariectomized animals bearing an implant of 17β-estradiol (E_2). $^{**}p < 0.01$, experimental versus OVX control rats.

The consequence of the partial agonistic activity of Tamoxifen is that "complete blockade of the action of estrogens cannot be achieved with Tamoxifen" [5]. It is thus 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.

8. Prevention of bone loss and inhibition of serum cholesterol and triglycerides

Osteoporosis is a disease characterized by a generalized loss of bone mass with the associated increased risk of fracture [177]. The reduction in circulating ovarian estrogen levels at menopause is thought to be largely responsible for the accelerated bone loss in women [178], and is also associated with the higher risk of coronary heart disease which is, at least partially, related to an increase in serum lipids [179,180]. The estrogen replacement therapy, commonly used to prevent or treat osteoporosis, reduces hot flushes and reduces the risk of coronary heart disease in postmenopausal women [181], but it requires the addition of progestins to counteract the endometrial proliferation induced by estrogens [182]. Moreover, some undesirable effects are associated with chronic estrogen and progestin administration, including a perceived increased risk for uterine and/or breast cancer [183–

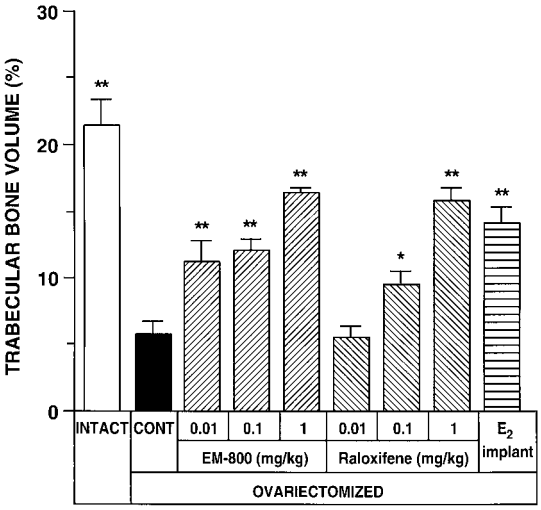

Fig. 28. Effect of 37-week treatment with increasing daily oral doses of EM-800 or Raloxifene on trabecular bone volume in ovariectomized rats. Comparison is made with intact rats. $^{**}P < 0.01$, experimental versus OVX control rats.

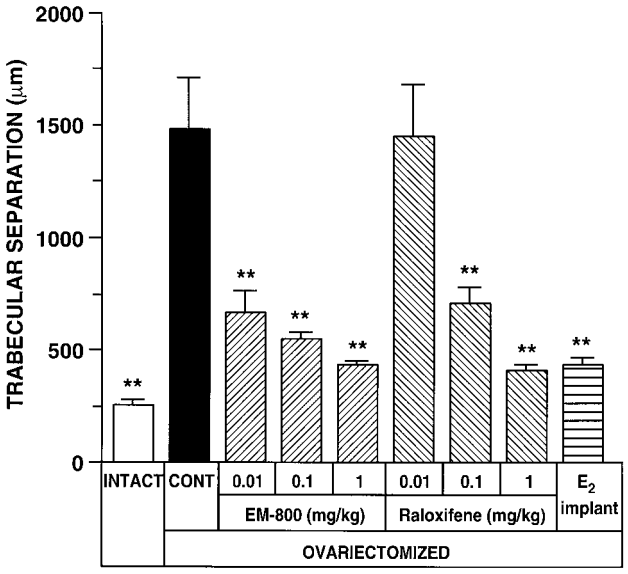

Fig. 29. Effect of 37-week treatment with increasing daily oral doses of EM-800 or Raloxifene on trabecular bone separation in ovariectomized rats. Comparison is made with intact rats. $^{**}P < 0.01$, experimental versus OVX control rats.

185]. Consequently, compliance with estrogen replacement therapy is low, thus indicating the need to develop novel approaches free of such risk.

Tamoxifen, an antiestrogen with partial agonistic properties, has been shown to maintain bone mass and lower serum cholesterol levels in postmenopausal women [186,187]. As indicated above, the uterotrophic activity of tamoxifen, however, is well documented, thus limiting its acceptability for the prevention and treatment of osteoporosis. In a previous study in rats, we have observed that the addition of EM-800 to dehydroepiandrosterone (DHEA) treatment showed an additive effect on many parameters of bone physiology, thus suggesting a positive action of EM-800 in bone [188].

The present report describes the ability of EM-800 to prevent bone loss and lower serum cholesterol levels in ovariectomized (OVX) rats and compares its effects with those of Raloxifene. The OVX rat is a well recognized animal model that mimics the development of estrogen deficiency-induced osteopenia in humans. It is also a useful model to study the lipid profile of compounds [190], a close parallel being found between the effect of selective estrogen receptor modulators (SERMs) as inhibitors of serum cholesterol and prevention of bone resorption [189,191]. Increasing doses of EM-800 and Raloxifene were thus administered orally for 37 weeks to OVX animals and the effect of these two compounds as well as that of 17β-estradiol (E_2) were examined on parameters of bone physiology and serum lipids.

Despite its pure antiestrogenic activity in the mammary gland and endometrium summarized above, EM-652 can be classified as a selective estrogen receptor modulator (SERM), as originally proposed for Raloxifene [192,193]. EM-652, however, is unique among SERMs in having pure antiestrogenic activity in both human breast and uterine cells [56,57,70,71] while being the most potent SERM studied so far in the prevention of loss of bone mineral density and as a lowerer of serum cholesterol in the rat.

It is of interest that studies with estrogens have shown that the inhibition of bone turnover found in short-term studies translates into increased bone mineral density (BMD) and decreased fracture rate in long-term studies [194–197]. The "estrogen-like" action of SERMs in the bone should thus lead to a decrease in bone fractures.

The mean pretreatment values of bone mineral density (BMD) measured in vivo by DEXA during the acclimation period at the lumbar spine, total body skeleton and femoral site were 0.148 ± 0.003 g/cm^2, 0.118 ± 0.001 g/cm^2 and 0.245 ± 0.004 g/cm^2, respectively. The effect of 35 weeks of treatment with increasing daily oral doses of EM-800 or Raloxifene on lumbar spine BMD is illustrated in Fig. 27. BMD of the lumbar spine was 19% lower in OVX control rats than in intact controls ($p < 0.01$). The animals given EM-800 or Raloxifene at doses of 0.01–1 mg/kg had 90–93% and 85–90%, respectively, of the BMD observed in intact rats, the BMD values being significantly higher than those of OVX control rats ($p < 0.01$), with the exception of the lowest dose of Raloxifene (0.01 mg/kg) which did not have a statistically significant effect on this parameter. Lumbar spine BMD of rats treated with 17β-estradiol (E_2) was 92%

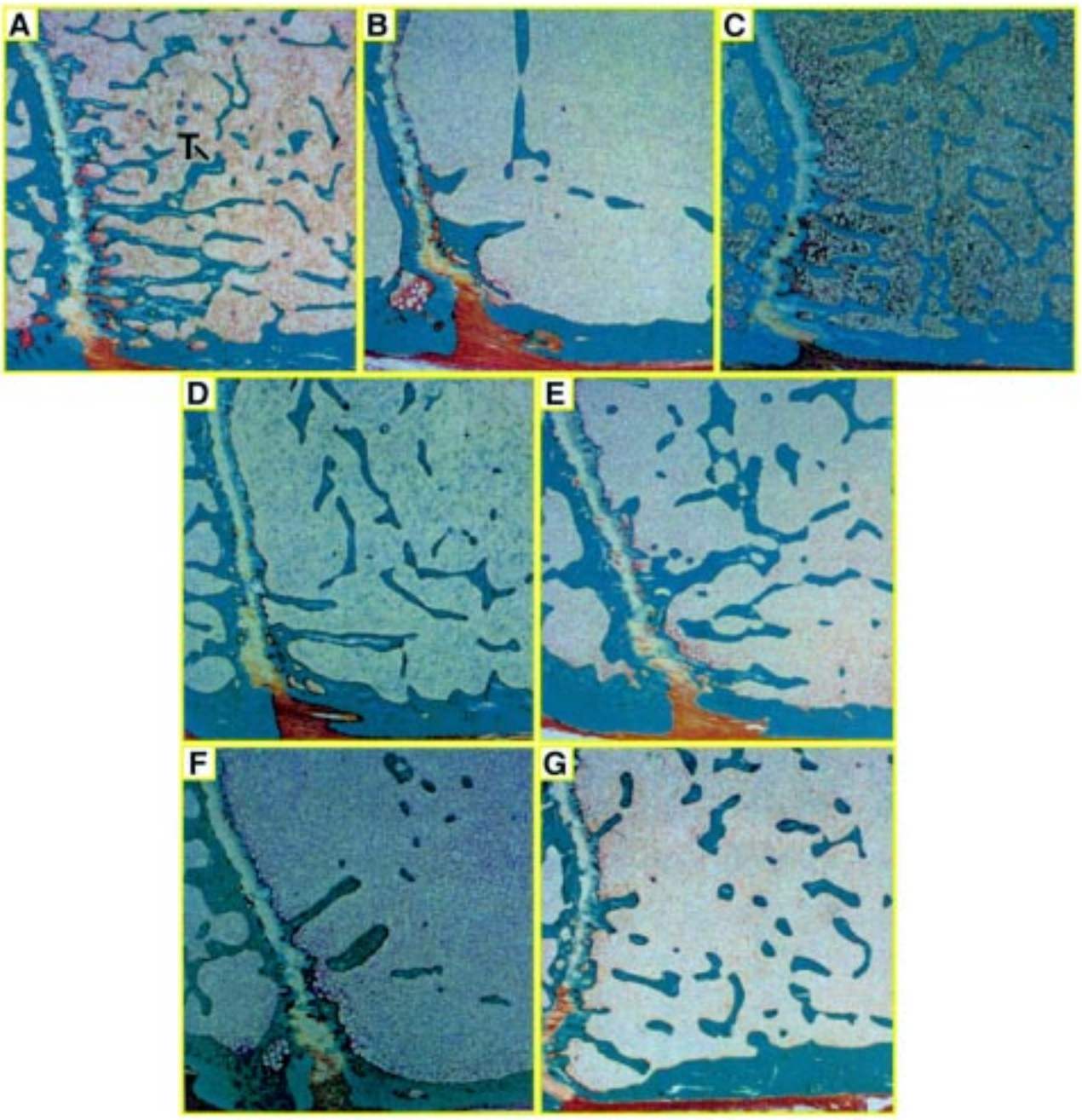

Fig. 30. Proximal tibia metaphysis from intact control (A), ovariectomized control (B), ovariectomized animals bearing an implant of 17β-estradiol (C) and ovariectomized rats treated with 0.01 mg/kg (D) and 1 mg/kg (E) of EM-800 or 0.01 mg/kg (F) and 1 mg/kg of Raloxifene (G). Note the reduced amount of trabecular bone in ovariectomized control animals, and the significant prevention of trabecular bone volume after EM-800 (0.01 and 1 mg/kg) or Raloxifene (1 mg/kg) administration. Modified trichrome Masson–Goldner (magn. ×100). T: Trabeculae.

($p < 0.01$) of that observed in the intact controls. This stimulatory effect of E_2 is not statistically different from that of EM-800 at all doses studied. It is of interest to mention that EM-800 already had a maximal stimulatory effect on lumbar spine BMD at the lowest dose used (0.01 mg/kg BW, $p < 0.01$), while a statistically significant effect of Raloxifene was first observed at the 0.03 mg/kg BW ($p < 0.01$), thus indicating that EM-800 is at least three times more potent than Raloxifene on lumbar spine BMD.

Thirty seven weeks after ovariectomy, marked decreases of 73% ($p < 0.01$) and 77% ($p < 0.01$) in trabecular bone volume (Fig. 28) and trabecular bone number (data not shown), respectively, were observed at 1–5 mm of the growth plate metaphyseal junction of the proximal tibia. Simultaneously, a marked

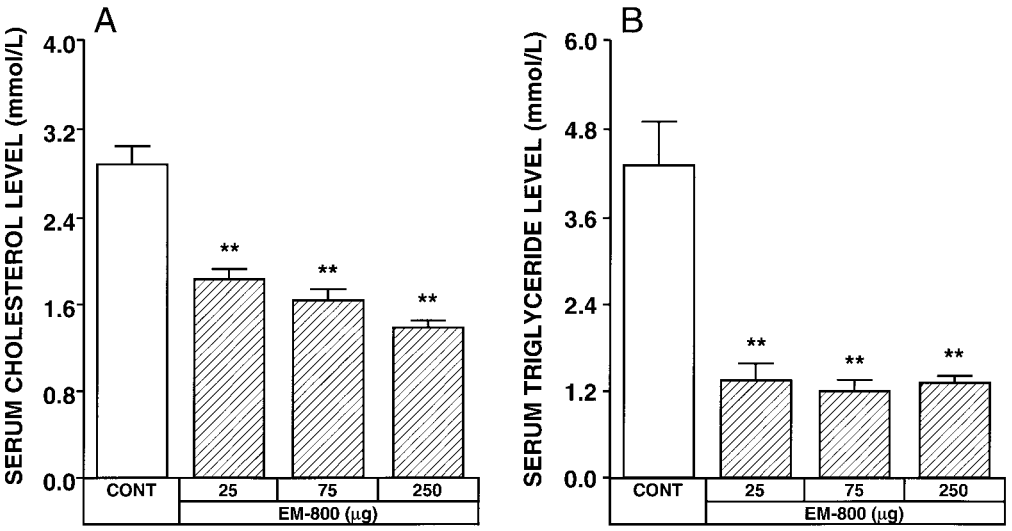

Fig. 31. Effect of daily oral administration of 25, 75, or 250 μg EM-800 for 9 months on serum cholesterol (A) and triglyceride (B) levels in the rat. The number of animals per group was 9, 14, 16, and 20, respectively. Data are expressed as the means ± SEM. $^{**}P < 0.01$ vs control [222].

increase in trabecular bone separation (Fig. 29) from a control value in intact rats of 262 ± 19 to 1486 ± 236 μm ($p < 0.01$) was observed in OVX animals. Treatment with 1 mg/kg of EM-800 and Raloxifene resulted in 68% ($p < 0.01$) and 64% ($p < 0.01$) reversals of the decrease in trabecular bone volume (Fig. 28) caused by ovariectomy, respectively. In fact, treatment with EM-800 and Raloxifene at the daily 1 mg/kg dose increased trabecular bone volume of the proximal tibia from a control value of 5.8 ± 0.9% in OVX animals to 16.4 ± 0.4% and 15.8 ± 1.0%, respectively. These stimulatory effects are not statistically different from the reversal achieved with E_2. At the lowest dose used (0.01 mg/kg), EM-800 already reversed by 34% ($p < 0.01$) the effect of OVX while Raloxifene had no detectable effect. The administration of 0.1 mg/kg of EM-800 and Raloxifene, on the other hand, resulted in 40% ($p < 0.01$) and 24% ($p < 0.05$) reversals of the decrease in trabecular bone volume (Fig. 28) caused by OVX, respectively.

It can be seen in Fig. 29 that the 0.01 mg/kg dose of EM-800 already caused a 66% ($p < 0.01$) reversal of the effect of OVX while a 76% ($p < 0.01$) reversal of this parameter was observed at the 0.1 mg/kg dose. Raloxifene, on the other hand, had no detectable effect at the lowest dose used (0.01 mg/kg) while a 63% ($p < 0.01$) reversal of the effect of OVX was observed at the 0.1 mg/kg dose. At the 1 mg/kg dose, EM-800 and Raloxifene caused 85% ($p < 0.01$) and 88% ($p < 0.01$) decrease in trabecular bone separation, compared to OVX controls. E_2, on the other hand, reversed by 85% ($p < 0.01$) the effect of OVX, a value similar to that achieved with the 1 mg/kg dose of EM-800 or Raloxifene.

Fig. 30 illustrates the prevention of trabecular bone volume loss in the proximal tibial metaphysis induced by EM-800 and Raloxifene in ovariectomized treated animals compared to OVX controls (B). The administration of 0.01 mg/kg of EM-800 (D) already prevented by 52% the OVX-induced osteopenia while Raloxifene had no detectable effect at the same dose (F). Treatment with 1 mg/kg of EM-800 or Raloxifene

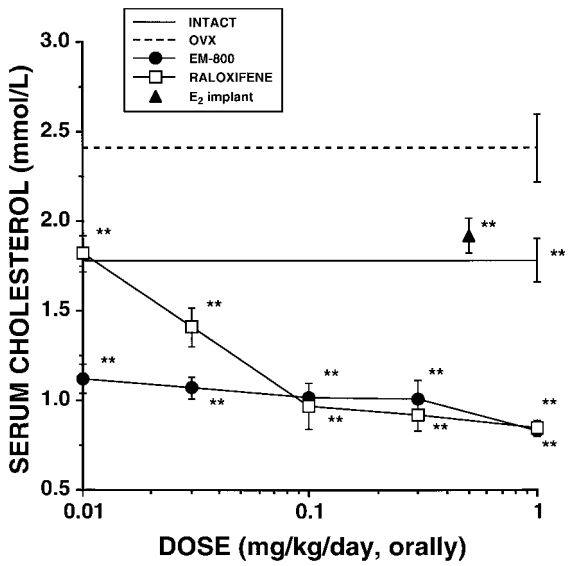

Fig. 32. Effect of 37-week treatment with increasing daily oral doses of EM-800 or Raloxifene on total serum cholesterol levels in ovariectomized rats. Comparison is made with intact rats and ovariectomized animals bearing an implant of 17β-estradiol (E2). $^{**}P < 0.01$, experimental versus OVX control rats.

(E, G) resulted in an approximately 75% prevention of the ovariectomy-induced osteopenia.

9. Effects of EM-800 on serum cholesterol and triglyceride levels

As can be seen in Fig. 31, a 36% reduction of serum cholesterol was already observed with the lowest dose of EM-800 used, the serum cholesterol concentration being already decreased from 2.9 ± 0.18 mmol/L to 1.8 ± 0.09 mmol/L at the daily 25 μg dose of EM-800 ($p < 0.01$). The daily 75 μg dose of EM-800 further decreased serum cholesterol to 1.6 ± 0.12 mmol/L ($p < 0.01$) while the 250 μg dose of EM-800 caused a maximal inhibition of 52% to a value of 1.4 ± 0.06 mmol/L ($p < 0.01$). The 250 μg dose had an inhibitory effect significantly ($p < 0.01$) more important than that of the 25 μg dose of EM-800, while the 75 μg dose had an intermediate inhibitory effect not significantly different from that of the 25 and 250 μg doses.

A similar inhibitory effect of EM-800 was observed on serum triglyceride levels. The daily administration of 25 μg of EM-800 for 9 months induced a near-maximal inhibition (69%) of serum triglyceride levels, which were measured at 1.4 ± 0.21 mmol/L ($p < 0.01$) while the value in control animals was 4.3 ± 0.62 mmol/L. Daily oral administration of 75 μg of EM-800 caused a maximal inhibition (72%) of serum triglyceride levels to 1.2 ± 0.15 ($p < 0.01$) mmol/L while the 250 μg dose of EM-800 decreased serum TG levels to 1.3 ± 0.12 mmol/L ($p < 0.01$). There was no statistical difference between the inhibitory effect of the three doses of EM-800.

We also compared the effect of increasing doses of EM-800 and Raloxifene on serum cholesterol levels (Fig. 32). Thirty-seven weeks after ovariectomy, a 35% increase ($p < 0.01$) in serum cholesterol was observed in OVX control rats compared to intact controls. The daily oral administration of 0.01 and 0.03 mg/kg of EM-800 to OVX animals already caused respective 54% ($p < 0.01$) and 56% ($p < 0.01$) reduction of serum cholesterol levels relative to OVX controls while Raloxifene administered at the same doses caused respective 24% ($p < 0.01$) and 41% ($p < 0.01$) decreases of the value of the same parameter. When administered at the daily doses of 0.1, 0.3 and 1 mg/kg, EM-800 caused respective 58%, 58% and 66% (all $p < 0.01$ versus OVX control rats) inhibitions of serum cholesterol levels while Raloxifene caused respective 60%, 62% and 65% decreases of this parameter at the same doses (all $p < 0.01$). The estradiol implant (E_2), on the other hand, only reduced serum cholesterol by 20% ($p < 0.01$) compared to OVX control rats.

The present data show that EM-800 produces marked hypocholesterolemic and hypotriglyceridemic effects in the rat, thus suggesting the possibility of additional beneficial effects in women. It has been repeatedly reported that estrogens and some antiestrogen compounds lower serum cholesterol levels in the rat [189,198–205] as well as in the human [179,186,206–209]. The effect of estrogens on human and rat high density lipoproteins (HDL) have been found to be opposite, with an usual increase in serum HDL levels in the human [208,209], but a decrease in the rat [202,204,210]. On the other hand, estrogens are known to elevate serum triglyceride levels in both the rat [198,200] and human [179,186,207–209], thus demonstrating a potential adverse effect on lipid metabolism.

It is thus of particular interest to see that EM-800 reduces both serum cholesterol and triglyceride levels, thus indicating a potential more global beneficial effect of EM-800 on lipid metabolism. The other antiestrogens, such as tamoxifen [145,186,198,207], droloxifene [199], and raloxifene [189], have been reported to elicit beneficial effects on the serum lipid profile, but they have not demonstrated such effect on serum triglycerides in the rat or in the human. The ability to lower both serum cholesterol and triglyceride levels seems to be unique to EM-800. The similar inhibitory effect achieved with the 25, 75 and 250 μg doses of EM-800 on serum triglyceride levels suggests a higher sensitivity of this parameter to the action of EM-800 compared to serum cholesterol.

An ideal therapy at menopause should prevent bone loss and, simultaneously, reduce cardiovascular risks without producing significant estrogenic effects on the endometrium and mammary gland which seriously limit the acceptance of the current estrogen replacement therapy. EM-800 lacks a stimulatory effect on the endometrium as shown at histopathological examination in this study as well as in previous ones [72, Sourla, 1997 no. 2694, 211]. Similarly, EM-800 shows pure antiestrogenic activity in human endometrial Ishikawa carcinoma cells [57]. Raloxifene, on the other hand, has been shown to have no stimulatory effect on the endometrium on short-term study in rat [189]. However, in the present long-term study, Raloxifene causes a significant stimulation of the endometrial epithelium at doses which are effective to prevent bone loss (0.1–1 mg/kg). It should be mentioned also that Raloxifene, Droloxifene, and Tamoxifen stimulate, to various degrees, the estrogen-sensitive parameter alkaline phosphatase in human endometrial Ishikawa carcinoma cells, the stimulatory effect of these compounds being fully reversible by EM-800 [56,57].

The present data clearly demonstrate that, in the rat, low doses of EM-800 prevent bone loss and lower serum cholesterol levels without stimulatory effect on the endometrium while previous studies have described the pure antiestrogenic activity of this compound in the mammary gland. Such data are encouraging and

Table 2
Site(s) of disease at start of treatment and at failure to EM-800 therapy (second progression) in 43 Tamoxifen-failure patients

Site(s) of disease at start of EM-800	Number of patients	Best response achieved				Site of failure to EM-800 (all patients)
		CR	PR	NC	Pg (any site)	
Bone(s)	28	0	2	10	16	20
Node(s)	15	1	3	2	9	8
Liver	11	0	0	2	9	11
Skin	8	0	2	4	2	4
Lung	7	0	0	1	6	5
Breast	2	0	2	0	0	0
Parotid gland	1	0	0	0	1	1
1 organ site	20	1	2	8	9	
2 organ sites	17	0	2	3	12	
3 organ sites	6	0	1	2	3	

suggest that the antiestrogen EM-652 and its precursor EM-800 could have the potential of exerting simultaneous beneficial effects on four important aspects of woman's health, namely prevention and/or treatment of breast and uterine cancer, osteoporosis and coronary heart disease.

Although pure steroidal antiestrogens such as ICI 164384, ICI 182780 and EM-139 could also be more effective than Tamoxifen in controlling estrogen-sensitive breast cancer, they cannot prevent bone loss and might even have harmful effects on the cardiovascular system [212–215].

10. Response to EM-800 (SCH 57050) in Tamoxifen-resistant breast cancer

In order to test the hypothesis that a more specific and more potent antiestrogen devoid of estrogenic activity in human breast [56,65,70] and endometrial [57] cancer cells could show increased clinically efficacy, we have administered the novel antiestrogen EM-800 in women who had failed Tamoxifen therapy. This approach is supported by the finding that human breast cancer cell lines showing resistance to Tamoxifen retain their sensitivity to pure antiestrogens under in vitro conditions [141,145–147] and as xenografts in nude mice [71,140,148,149].

Forty-three (43) postmenopausal or ovariectomized women of a median age of 67 years (43–86 years) with breast cancer resistant to Tamoxifen were treated with the daily oral doses of 20 or 40 mg of EM-800 (SCH-57050) (Fig. 1).

The patients had progressive metastatic or locally advanced inoperable biopsy- or fine needle aspiration-proven breast cancer that had responded to Tamoxifen (complete or partial response) or stable disease for at least 6 months but were in progression. Patients originally treated with Tamoxifen as adjuvant to surgery for at least 1 year who were in progression under Tamoxifen or who were progressing after cessation of Tamoxifen were also candidates. Tamoxifen had to be stopped for at least one month unless the investigator judged that the disease was rapidly progressing where 2 weeks were sufficient before starting treatment with EM-800. The study was approved by the Institutional Review Board (IRB) of each hospital or university.

As summarized in Table 2, the predominant sites of failure to Tamoxifen at start of EM-800 administration were in decreasing order of incidence rate: bone (28), nodes (15), liver (11), skin (8), lung (7), breast (2), and parotid gland (1). Progression was present at one site in 20 patients, at two sites in 17 patients and at three sites in 6 patients at start of treatment with the antiestrogen.

As shown in Table 3, one (1) patient had a complete response and is still responding at 27 months while 5 patients had a partial response. Complete (CR) and partial (PR) responses have thus been observed so far in 6 patients (13.9%) while NC (No Change for at least 3 months) has been observed in 13 patients (30.2%), for a total of 19 positive responses out of 43 evaluable patients (44.2%). When stable response for at least 6 months is considered, 10 patients meet this criteria for a total of 16 positive objective responses out of 43 patients or 37.2%. Four patients are still responding, namely one at 27 months (CR), one at 21 months (PR) and two at 23 and 24 months of stable disease, respectively (Table 3). The CR was observed after 3 months of treatment in a patient who had shown progression in a node of the right axilla while being treated with Tamoxifen as adjuvant for 42 months. Of the 5 PR responders, one patient had received Tamoxifen for advanced disease for 8 months while four had received adjuvant therapy for 5, 61, 64 and 108 months, respectively. In the NC category, two had received adjuvant Tamoxifen for only 13 and 14 months while two had received adjuvant Tamoxifen

Table 3
Best response achieved in Tamoxifen-resistant advanced or locally inoperable breast cancer patients treated with the orally active specific antiestrogen EM-800 (SCH-57050). Letters between parentheses indicate Tamoxifen administered for advanced disease (P, palliative) and/or as adjuvant to surgery (A, adjuvant). The numbers indicate the duration of each treatment in months; +, Response still ongoing

	Duration of response (months)	
Type of response	20 mg (EM-800)	40 mg (EM-800)
CR[a]		27 + (A42)
PR[b]	15(A61), 9(A108), 21 + (A64)	9(P8), 8(A5)
NC[c]	6(A37), 4.5(A14), 9(A70), 11(P26), 9(A24P40)	9(A13), 17(A60P32), 4.5(P25), 24 + (A73P2), 23 + (P92), 16(P64), 4(P10), 6(P34),
Pg[d]	1(P22), 2.5(A25), 2(P17), 1(A57), 3(A58), 1(P16), 1(A42P6), 3(P23), 1(A115), 3(P35), 3(P38), 1(A14), 3(A60)	1.5(A46), 1(A17P6), 1(P27), 1(P24), 3(P10), 3(A26), 3(A36), 1(A13), 3(A58), 2(A31), 1(A74P3)

[a] CR: complete response.
[b] PR: partial response.
[c] NC: no change.
[d] Pg: progressive disease.

for 37 and 70 months, respectively. In the same category of response, six had received palliative treatment for advanced disease for 10, 25, 26, 34, 64 and 92 months, respectively, while three had received Tamoxifen for both adjuvant therapy and advanced disease. In the group of patients who showed no positive response to EM-800, twelve had received adjuvant Tamoxifen only for 13–115 months, three had adjuvant followed by palliative Tamoxifen while nine had received Tamoxifen for advanced disease for 10–38 months.

The present findings suggest that the progression of breast cancer which occurs under Tamoxifen treatment can be due to the intrinsic estrogenic stimulatory activity of Tamoxifen on breast cancer proliferation [27–29,31,32,37,39,72,140,141,145,146,148]. As mentioned above, clinical evidence supports the suggestion that Tamoxifen-stimulated tumor growth is a mechanism responsible for Tamoxifen resistance or no response in an unknown proportion of breast cancer patients [27,31,32,142–144]. The possibility also exists that changes in the intracellular metabolism or distribution of Tamoxifen could explain the loss of response to this antiestrogen [149,216]. In a small proportion of cases, the resistance to Tamoxifen could possibly be explained by loss of ER expression or ER mutation [140,217].

When LY156758 (Raloxifene) was used as second-line treatment in a group of 14 patients, no complete or partial response was found while five patients (36%) showed no change [218]. It is clear, however, that large-scale and randomized studies are required to truly assess the benefits of a new drug in such an heterogeneous and difficult-to-treat population of patients having shown failure to Tamoxifen.

Due to its specific 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 estrogen-related risk of carcinogenicity [219] and induction of uterine carcinoma [220] during long-term use in women. In fact, 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 [57]. In long-term studies in the rat, mouse, and monkey, EM-800 shows a potent inhibitory effect on the uterus [123, 221, Labrie et al., unpublished data]. Moreover, the new antiestrogen EM-800 has been shown to have good oral bioavailability in the mouse, rat, monkey, and human, thus providing an important advantage over the steroidal antiestrogens which possess poor oral bioavailability [19,139].

In toxicology studies using up to 25 mg/kg body weight, a dose approximately 35-fold higher than the

highest dose used in the present study, no toxic effects other than the endocrine changes expected from a pure antiestrogen have been observed in female rats and monkeys treated daily for 6 months. In phase I studies where 145 normal post-menopausal women received daily doses of EM-800 up to 40 mg for up to 14 days as well as in this phase II study where breast cancer patients received the daily 40 mg dose up to more than 2 years, no serious adverse effect related to the drug has been observed.

Strongly supported by a long series of preclinical studies, the present data suggest that EM-800 (SCH-57050) may improve the rate, quality and duration of response of advanced breast cancer to endocrine therapy. Further studies are required to obtain a more precise assessment of the response rate and its duration and to determine the long-term effects on bone, lipids, and the endometrium. It is of interest that this compound has been shown to prevent bone loss in the ovariectomized rat (Fig. 27) and to decrease serum cholesterol and triglycerides in the rat [222] and in postmenopausal women (Labrie et al., unpublished data). Coupled with its pure antiestrogenic activity in human breast and endometrial cancer cells, the lack of toxicity seen in any phase I and II study in women as well as in all the toxicology studies performed in the rat and monkey, the present data suggest the interest of studying the effect of EM-800 (SCH-57050) or its active compound EM-652 (SCH 57068) in the neoadjuvant and adjuvant settings and, most importantly, for prevention of breast and uterine cancer.

As predicted by detailed preclinical studies, the present clinical data obtained in Tamoxifen-failure patients suggest that EM-800 (SCH-57050) is a promising new drug for the prevention and treatment of breast and endometrial cancer while exerting beneficial effects on bone and lipids.

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