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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

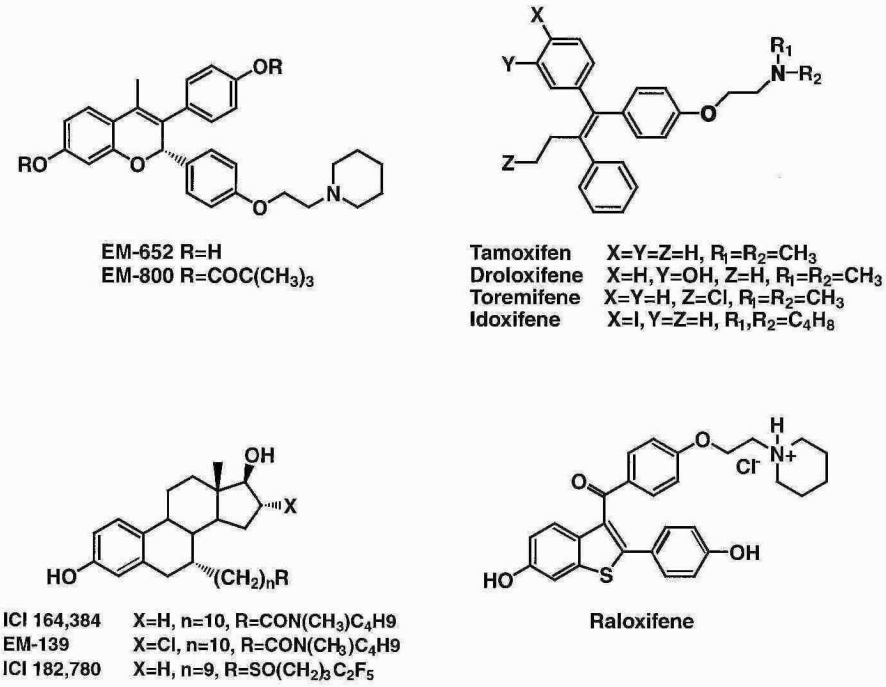

Fig. 1. Antiestrogens—molecular structures.

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-

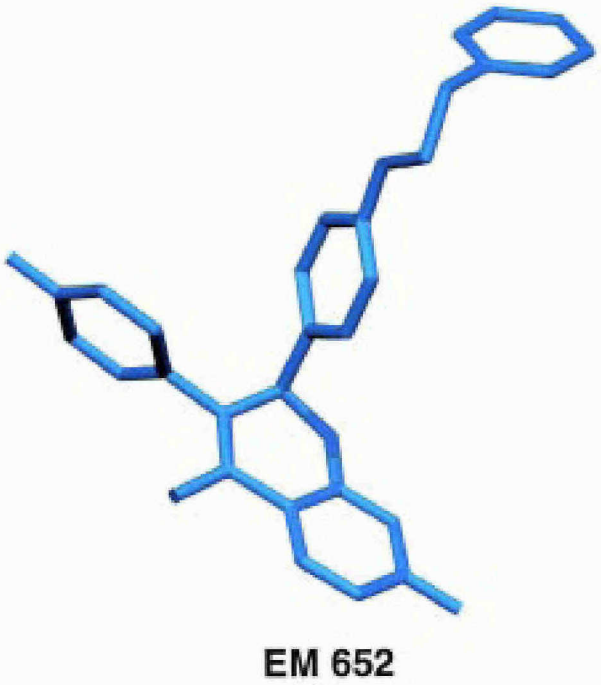

Fig. 2. Structure of EM-652 (SCH 57068).

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

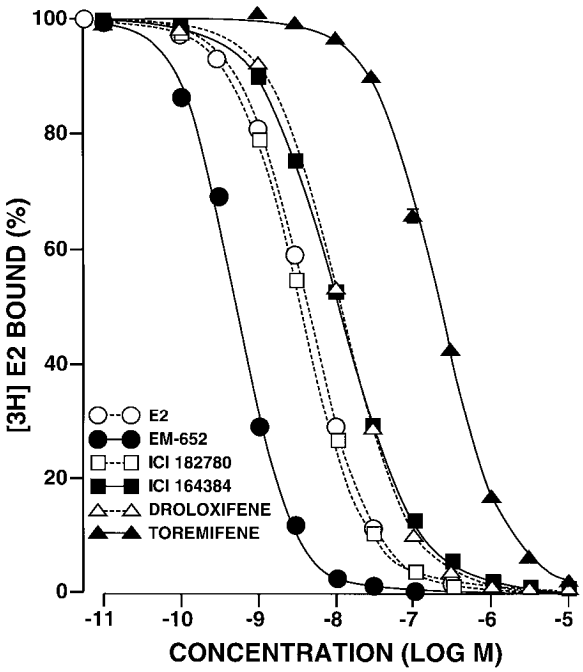

Fig. 3. Effect of increasing concentrations of EM-652, E2, ICI 182780, Droloxifene, ICI 164384, and Toremifene on [^{3}H] 17β-estradiol binding to the rat uterine estrogen receptor. The incubation was performed with 5 nM [^{3}H] 17β-estradiol (E_2) for 2 h at room temperature in the presence or absence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

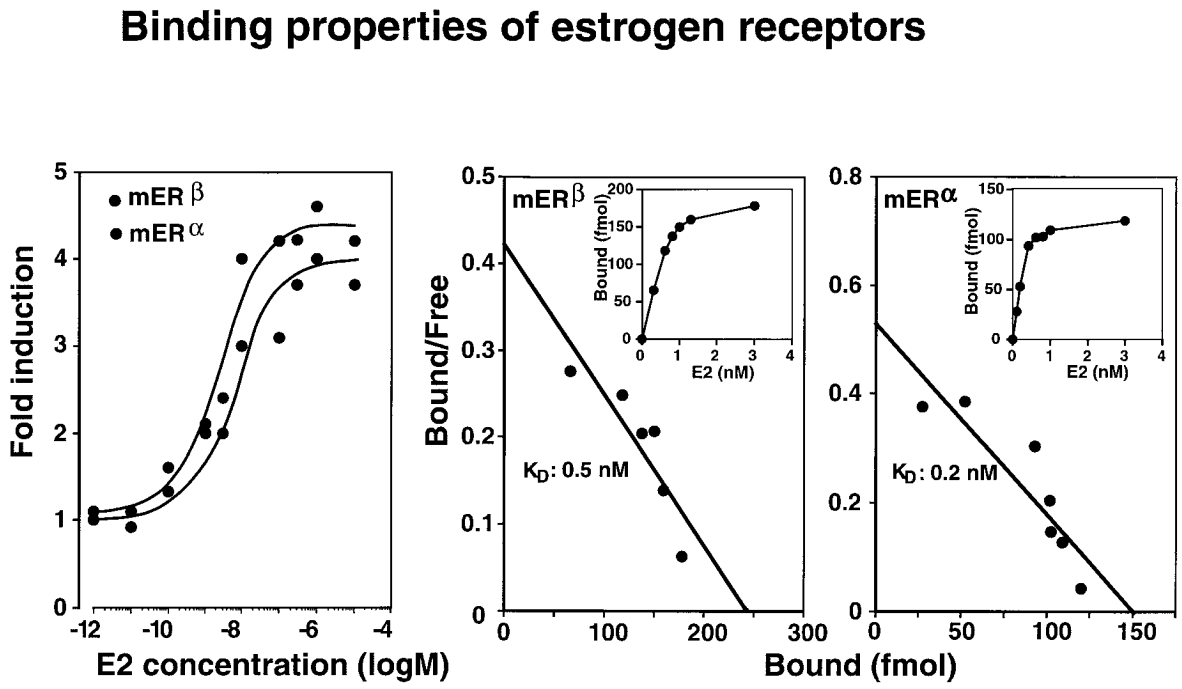

Fig. 4. Dose-response and binding properties of mERα and mERβ. (A) Cos-1 cells were transfected with 500 ng mERβ (open circles) or mERα (closed circles) expression vectors and 1 μg vitA$_2$-ERE-TKLuc and then incubated for 12 h with increasing concentrations of E_2 as indicated. (B) Specific binding 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

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