

LONG-TERM INHIBITORY EFFECTS OF A NOVEL ANTI-ESTROGEN ON THE GROWTH OF ZR-75-1 AND MCF-7 HUMAN BREAST CANCER TUMORS IN NUDE MICE

Shouqi LUO, Céline MARTEL, Sylvain GAUTHIER, Yves MÉRAND, Alain BÉLANGER, Claude LABRIE and Fernand LABRIE*

Laboratory of Molecular Endocrinology, CHUL Research Center, Quebec, Canada

The effects of the novel anti-estrogen EM-343 on the growth of 2 hormone-responsive human breast cancer tumors have been examined in athymic nude mice. At the low daily dose of 5 µg, EM-343 administered subcutaneously for 6 months completely blocked the stimulatory effect of endogenous estrogens on the growth of ZR-75-1 and MCF-7 tumors implanted in nude mice. In addition, uterine weight decreased by 60% while ovarian weight increased by 37%. Estrogen receptor (ER) levels measured by [³H]-labeled estrogen binding were markedly reduced (by 96%, 96% and 92%) in ZR-75-1 and MCF-7 tumors, and in the mouse uterus, respectively. Accompanying the decrease in ER, progesterone receptor levels were reduced by 79%, 87% and 76%, respectively, in the above-mentioned tissues following EM-343 treatment. Our data show the pure anti-estrogenic properties of EM-343 and its high potency as an inhibitor of growth of human ZR-75-1 and MCF-7 breast tumors in nude mice. *Int. J. Cancer* 73:735–739, 1997.

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It is estimated that 181,600 new cases of breast cancer will be diagnosed, while 43,900 women will die from this disease in the United States alone in 1997 (Parker *et al.*, 1997). In fact, breast cancer accounts for 30% of new cancer cases in women and for 17% of all female cancer deaths.

Tamoxifen has become the standard treatment for breast cancer at all stages of the disease in both pre-menopausal and post-menopausal women (Fisher *et al.*, 1996; Furr and Jordan, 1984; Lerner and Jordan, 1990; Swedish Breast Cancer Cooperative Group, 1996). Its potential benefits for the prevention of breast cancer is under clinical investigation (Powles *et al.*, 1989). Tamoxifen, however, possesses partial estrogenic activity in breast cancer tissue (Gottardis *et al.*, 1989), thus limiting its therapeutical potential for the treatment of breast cancer in women. In fact, about 40% of the patients do not respond to the anti-estrogen despite the presence of estrogen receptor in malignant tissues (Kawamura *et al.*, 1989). Moreover, the positive response is usually of short duration (Mouridsen *et al.*, 1978). The availability of pure antagonists of estrogen action offers the possibility of improving the treatment of breast cancer (Dauvois *et al.*, 1991; Labrie *et al.*, 1992; Wakeling, 1993b). Such compounds should achieve more complete inhibition of tumor growth, increase cancer cell death or apoptosis, prolong tumor response, avoid tumor flare and eliminate the risk of endometrial cancer due to the partial estrogenic activity of the mixed agonist-antagonist anti-estrogens.

The present study reports on the anti-tumoral activity of a new anti-estrogen, EM-343, and its characteristics of pure antagonist of estrogen action in nude mice bearing xenografts of human ZR-75-1 and MCF-7 breast cancer tumors.

MATERIAL AND METHODS

Human breast cancer cell lines

ZR-75-1 and MCF-7 human breast cancer cells were obtained from the ATCC (Rockville, MD). ZR-75-1 cells were routinely cultured in phenol red-free RPMI-1640 (Poulin and Labrie, 1986) while MCF-7 cells were grown in phenol red-free Dulbecco's modified Eagle's-Ham's F-12 medium (DME-F-12) (Simard *et al.*, 1990). Both media were supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU penicillin/ml, 100 µg streptomycin/ml and 10% (vol/vol) fetal bovine serum and incubated in a humidified atmosphere of 95% air/5% CO₂ at 37°C. Cells were passaged weekly by treatment with 0.05% trypsin/0.02% EDTA (w/v). The cells used for the present experiments were from passages 50 to 60 for ZR-75-1 cells and from passages 100 to 110 for MCF-7 cells.

Animals and tumor inoculation

Female homozygous BALB/C (*nu/nu*) athymic mice (28–42 days old) were obtained from Charles River Canada (St. Constant, Canada). The mice were housed in vinyl cages with air filter tops in laminar air flow hoods and maintained under pathogen-limited conditions. Cages, bedding, food and water were autoclaved before use. Water was acidified to pH 2.8 and was available *ad libitum*.

Animals and tumor inoculation

Cells (2×10^6) in their logarithmic growth phase harvested with 0.05% trypsin/0.02% EDTA (w/v) were inoculated s.c. in 0.1 ml of medium on the flanks of the mice through 1- to 2-cm-long 20-gauge needles. ZR-75-1 cells were inoculated on the left side while MCF-7 cells were seeded on the right flank. Silastic implants (0.5-cm-long with an inner diameter of 0.15 cm and an outside diameter of 0.23 cm) filled with 1/750 estradiol (estradiol/cholesterol, w/w) were placed in the intercapsular region of the animals under Avertin-induced anesthesia, 1 week before inoculation of the cancer cells to facilitate tumor growth. The implants were removed 2 weeks before starting treatment.

Treatment

EM-343 (7-hydroxy-3-(4'-hydroxyphenyl)-4-methyl-2-(4''-2''-piperidinoethoxy) phenyl)-2H-benzopyran) synthesized in the medicinal chemistry division of the Laboratory of Molecular Endocrinology was dissolved in 5% ethanol/1% gelatin 0.9% NaCl. EM-343 is an approximately 50:50 mixture of the active enantiomer EM-652 and its corresponding inactive enantiomer (EM-651) (Gauthier *et al.*, 1997; Luo *et al.*, 1997; Simard *et al.*, 1997). Mice bearing tumors with diameters ranging from 0.4 to 0.7 cm were selected for this study. The mice were then randomly assigned to groups each containing 15 animals. Tumors were then measured once a week with calipers. Two perpendicular diameters were recorded and tumor area (cm²) was calculated using the formula: $L/2 \times W/2 \times \pi$ (Dauvois *et al.*, 1989). The area measured on the first day of treatment was taken as 100% and change of tumor growth was expressed as percentage of initial tumor area. Plasma was kept at -20°C until assayed. At sacrifice, tumors, uteri and ovaries were immediately removed, freed from connective and adipose tissue, weighed, frozen in liquid nitrogen and stored at -80°C until assayed.

Treatment

Steroid receptor assays

Preparation of cytosol. Tumors or uteri were homogenized with a PT-10 homogenizer (Brinkman, Mississauga, Canada) at a setting of 8 for 10 sec in 20 vol of buffer A (25 mM Tris-HCL, 1.5 mM EDTA, disodium salt, 10 mM α -monothioglycerol, 10% glycerol

Steroid receptor assays

*Correspondence to: MRC Group in Molecular Endocrinology, CHUL Research Center, 2705 Laurier Boulevard, Québec, Québec, G1V 4G2, Canada. Fax: (418) 654-2735. E-mail: fernand.labrie@crchul.ulaval.ca

and 1.5 mM sodium molybdate, pH 7.4). The homogenates were then centrifuged at 105,000g for 60 min at 4°C. Steroid binding assays were performed with freshly prepared cytosol. Protein concentration was measured by the method of Bradford (1976) using BSA as standard.

Progesterone and estrogen receptor assays. 6,7[³H]-17,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione (R5020) (87 Ci/mmol), [³H]-estradiol (115 Ci/mmol) and unlabeled R5020 were from New England Nuclear (Boston, MA) while diethylstilbestrol (DES) was purchased from Sigma (St. Louis, MO). [³H]-R5020 or [³H]-estradiol binding was measured using the dextran-coated charcoal absorption technique (Asselin and Labrie, 1978). In brief, 0.2-ml aliquots of cytosol were incubated with 0.1 ml [³H]-R5020 (200,000 cpm, 8 nM, final concentration) and dexamethasone (120 nM, final concentration) or 0.1 ml [³H]-E₂ (90,000 cpm, final concentration = 3 nM) in the presence or absence of a 100-fold excess of unlabeled R5020 overnight at 4°C or a 100-fold excess of DES for 3 hr at room temperature. Unbound steroids were separated by incubation for 15 min at 4°C with 0.3 ml of 0.5% Norit A, 0.05% Dextran T-70 (DCC) in buffer B (1.5 mM EDTA, disodium salt, 10 mM α-monothioglycerol and 10 mM Tris-HCL, pH 7.4) and centrifugation at 3,000g for 15 min. Aliquots of supernatant (0.3 ml) were then taken for radioactivity measurement with 10 ml of scintillation fluid.

Statistical analysis

Statistical significance was calculated according to the test of Duncan-Kramer (Kramer, 1956). All data are presented as means ± SEM.

RESULTS

Inhibitory effect on the growth of ZR-75-1 and MCF-7 tumors in nude mice

As shown in Figure 1a, the growth of ZR-75-1 tumors was completely inhibited up to the last time interval studied by treatment with the low daily 5 µg s.c. dose of the anti-estrogen while tumors in the control group grew steadily up to 183 ± 18% of their initial size at the end of the 6-month observation period.

As illustrated in Figure 1b, a similar inhibitory effect was observed on the growth of MCF-7 human breast cancer tumors implanted in the same mice. The MCF-7 tumors grew to 243 ±

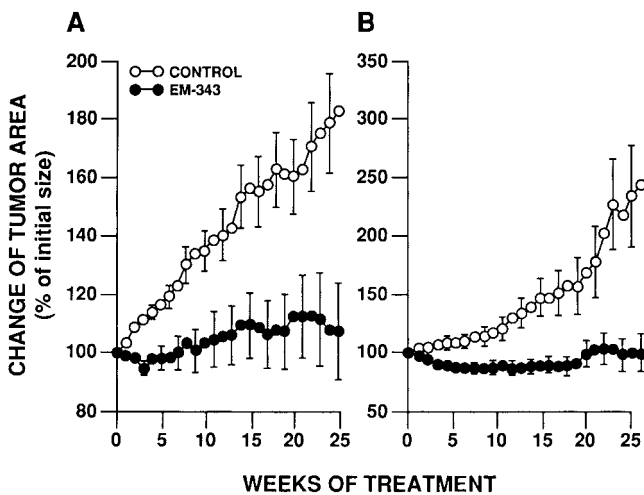


FIGURE 1 – Inhibitory effect of treatment for 6 months with EM-343 at the once daily s.c. dose of 5 µg on the growth of ZR-75-1 (a) and MCF-7 (b) human breast cancer tumors in intact nude mice. The average tumor area at start of treatment was 0.30 ± 0.02 cm² and 0.25 ± 0.01 cm² for ZR-75-1 and MCF-7 xenografts, respectively. Data are expressed as means ± SEM.

47% of their original size in intact control mice while the tumors in animals treated with EM-343 remained stable up to the end of the 6-month experiment. Administration of the low daily 5 µg dose of EM-343 can thus completely prevent the stimulatory effect of endogenous estrogens on the growth of MCF-7 as well as ZR-75-1 human breast cancer xenografts. In agreement with the above-described growth curves estimated by measurement of the tumors with calipers, EM-343 inhibited the weight of both ZR-75-1 and MCF-7 tumors measured at the end of the experiment from 241 ± 33 mg and 282 ± 84 mg in intact control animals to 104 ± 37 mg ($p < 0.05$), and 70 ± 28 mg ($p < 0.05$) for ZR-75-1 and MCF-7 tumors, respectively, in EM-343-treated mice (Fig. 2).

Effect on mouse uterine and ovarian weights

As illustrated in Figure 3, uterine weight decreased by 60% from 80 ± 9 mg in control mice to 32 ± 1 mg ($p < 0.01$) in the group of animals treated with EM-343 while ovarian weight increased by 37% from 7.0 ± 0.4 mg to 9.6 ± 0.5 mg ($p < 0.01$) during the same period.

Effect on ER and PR levels

As can be seen in Figure 4, treatment with the non-steroidal anti-estrogen EM-343 caused a 92% fall in [³H]-estradiol binding

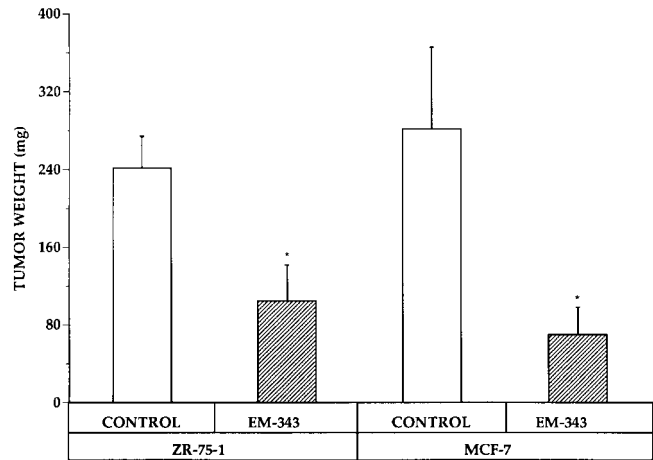


FIGURE 2 – Effect of treatment for 6 months with EM-343 at the once daily s.c. dose of 5 µg for on ZR-75-1 and MCF-7 tumor weight in nude mice. **, $p < 0.01$ vs. intact control. Data are expressed as means ± SEM.

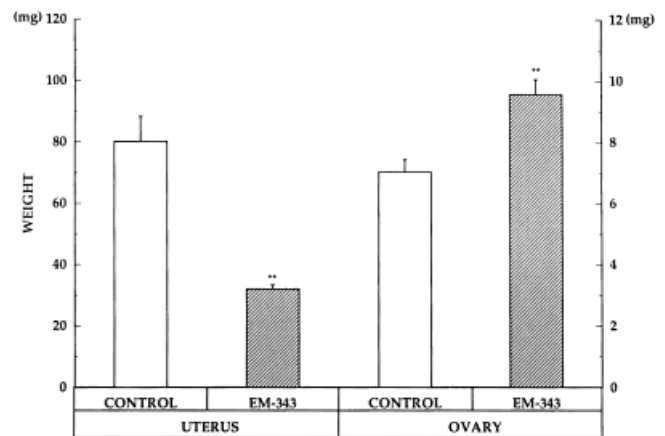


FIGURE 3 – Effect of treatment for 6 months with EM-343 at the once daily s.c. dose of 5 µg on uterine and ovarian weight in nude mice. **, $p < 0.01$ vs. intact control. Data are expressed as means ± SEM.

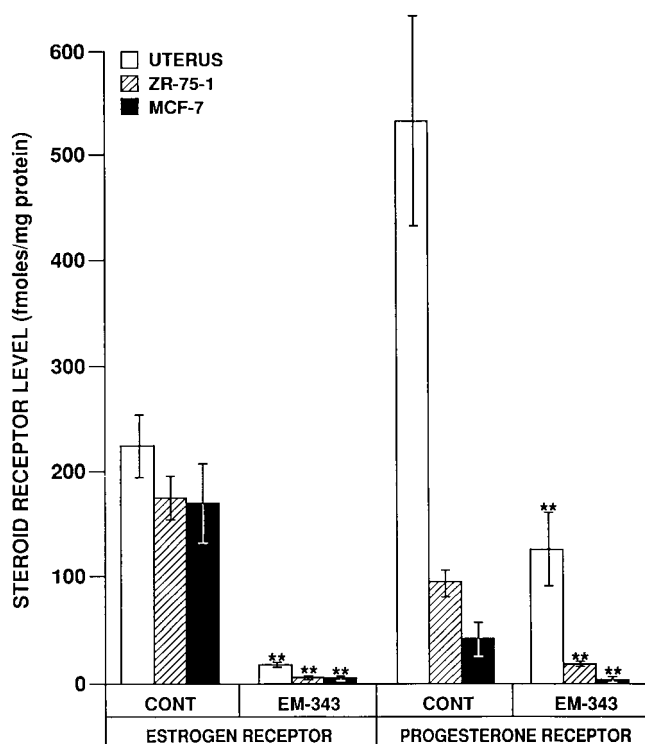


FIGURE 4—Effect of treatment for 6 months with EM-343 at the once daily s.c. dose of 5 μ g on estrogen and progesterone receptor levels in mouse uteri as well as ZR-75-1 and MCF-7 human breast cancer tumors in nude mice. Data are expressed as means \pm SEM. **, $p < 0.01$ vs. intact. Data are expressed as means \pm SEM.

in the uterine cytosol from 224 ± 30 to 18 ± 2 fmol/mg protein ($p < 0.01$). On the other hand, uterine progesterone receptor level decreased by 76% from 535 ± 100 to 126 ± 34 fmol/mg protein ($p < 0.01$). As illustrated in the same Figure, treatment with EM-343 caused a 96% fall in the levels of estrogen receptor in human breast cancer ZR-75-1 tumors after 6-month treatment, namely from 175 ± 21 to 6.0 ± 1.4 fmol/mg ($p < 0.01$), while the levels of progesterone receptors in the same tumors decreased by 79% from 95 ± 11 to 20 ± 0.7 fmol/mg protein ($p < 0.01$) during the same period. In MCF-7 tumors, the levels of estrogen receptor also fell markedly (96%) from 170 ± 37 to 5.9 ± 1.8 fmol/mg protein ($p < 0.01$) while the concentration of progesterone receptors fell by 89% from 41 ± 15 to 4.6 ± 3.4 fmol/mg protein ($p < 0.01$) after 6 months of treatment with EM-343.

DISCUSSION

Our data show the potent and long-term inhibitory effect of treatment with the new anti-estrogen EM-343 on the growth of 2 human breast cancer tumors xenografted in nude mice. Since EM-343 binds with high affinity to estrogen receptors, it can be suggested that EM-343 binds to estrogen receptors to form an ER-EM-343 complex that is either unable to bind to the estrogen-responsive element (ERE) (Farwell *et al.*, 1990) or, if binding to DNA occurs, the complex is unable to promote transcription (Reese and Katzenellenbogen, 1992).

Estrogens induce the expression of a series of genes including growth factors and growth factor receptors as well as some proteins such as collagenase and plasminogen activator, some of which are believed to enhance the metastatic capacity of breast cancer cells (Dickson and Lippman, 1987). Consequently, through an action at the first step of estrogen action, a pure anti-estrogen can be expected to inhibit these late or secondary effects resulting from

activation of the estrogen receptor, including growth factor formation and action. In other words, efficient blockade at the estrogen receptor level could be equivalent to an efficient blockade of all those largely unidentified secondary effects of estrogen receptor activation that lead to breast cancer cell proliferation. Anti-estrogens are also known to inhibit the synthesis and secretion of estrogen-induced autocrine growth factors (Vignon *et al.*, 1987). In addition, binding of an anti-estrogen to estrogen receptors enhances the production of some growth-inhibitory factors (Chalbos *et al.*, 1993).

Tamoxifen administration in women has been found to cause a reduction in serum insulin-like growth factor-1 (IGF-1) concentrations (Pollak *et al.*, 1992) through inhibition of serum growth hormone levels and inhibition of local production of IGF-1 at 2 sites of breast cancer metastasis, namely, the lung and liver (Huynh *et al.*, 1993). Anti-estrogens can also inhibit IGF-1 receptor concentrations (Freiss *et al.*, 1990), thus further reducing the activity of already reduced circulating levels of IGF-1. The role of these mechanisms of action in the potent inhibitory effects of EM-343 on human breast cancer tumor growth observed in the present study remains to be determined.

Our data show that EM-343 causes a marked decrease in estrogen receptor levels in the mouse uterus as well as in ZR-75-1 and MCF-7 tumors. To ensure that this down-regulation does not result from a failure of [3 H]-E $_2$ to exchange with EM-343 or the possibility that EM-343 occupies and masks estrogen receptors, we measured the content of estrogen receptors by enzyme immunoassay (ER-EIA, Abbott kit) and confirmed the down-regulation of the estrogen receptor protein (data not shown). In fact, in ZR-75-1 and MCF-7 human breast cancer tumors, the results obtained with both methods were comparable. However, in the mouse uterus, the values obtained with EIA were significantly higher than those obtained with the radioligand assay. The difference of estrogen receptor levels in the mouse uterus as measured by radio ligand assay (RLA) and ER-EIA could possibly be partially explained as follows: binding of EM-343 to the mouse estrogen receptor(s) could induce a conformational structural change leading to the exposure of an additional epitope, thus enhancing immunoreactivity toward the monoclonal antibody H222. Similar results were seen in human breast cancer cytosol following treatment with tamoxifen or 4-monohydroxytamoxifen (Martin *et al.*, 1988).

We have found that EM-652, the active enantiomer of EM-343, binds with high affinity to both murine ER α and ER β (Tremblay *et al.*, 1997). The presence of both ER α and ER β in the mouse uterus remains to be investigated, while only ER α seems to be expressed in at least some human breast cancer cell lines, including the ZR-75-1 and MCF-7 cell lines used in the present study (Kuiper *et al.*, 1997). It has also been found that the levels of estrogen receptor are reduced following exposure to the pure anti-estrogen ICI 164,384 in TM4 cells transiently expressing ERE (Dauvois *et al.*, 1992), a clonal non-tumorigenic cell line derived from mouse testis displaying the functional characteristics of Sertoli cells (Mather, 1980). Down-regulation of ER has also been found in the mouse uterus (Gibson *et al.*, 1991), this effect being explained by increased turnover of the estrogen receptor protein. In contrast, non-steroidal mixed agonist/antagonist anti-estrogens such as tamoxifen have little effect on estrogen receptor turnover (Eckert *et al.*, 1984).

Increased progesterone receptor synthesis is a late response to estrogenic stimulation (Horwitz and McGuire, 1978). Triphenylethylene anti-estrogens such as tamoxifen can induce expression of the progesterone receptor due to their partial estrogenic activity (Jordan and Dix, 1979). In contrast, pure anti-estrogens devoid of any estrogenic activity inhibit progesterone receptor expression. In the present study, the decrease in progesterone receptor levels after treatment with EM-343, which is parallel to the reduction in estrogen receptor levels in both ZR-75-1 and MCF-7 human breast cancer tumors, provides additional evidence of the pure anti-estrogenic activity of EM-343.

The major cause of death from breast cancer is metastatic disease rather than uncontrolled proliferation of the primary tumor. Metastatic spread depends on the invasive ability, which can be independent of the proliferative potential. Studies have shown that estradiol and 4'-hydroxytamoxifen stimulate the capacity of MCF-7 cells to cross a reconstituted basement membrane *in vitro* while ICI 164,384, a pure anti-estrogen, was able to block estrogen and 4-hydroxyl tamoxifen-stimulated invasion (Thompson *et al.*, 1989). ICI 164,384 has also shown anti-invasive activity in an alternative bioassay (Bracke *et al.*, 1991).

In patients with advanced breast cancer, responses to tamoxifen may last from several weeks to several years, with a median duration of 12–18 months. However, virtually all patients with advanced disease can expect to experience progression while still receiving tamoxifen (Muss, 1992). Several mechanisms have been suggested to explain the transformation from tamoxifen responsiveness to tamoxifen resistance: 1) mutation of the estrogen receptor (Horwitz, 1993); 2) formation of estrogenic metabolites *in vivo* (Osborne, 1993); 3) predominance of an autonomous, estrogen-independent clone as estrogen-dependent clones are inhibited (Shafit and Grantham, 1981); 4) selection of a sub-population of cells that read tamoxifen as an estrogen (Graham *et al.*, 1992); and 5) involvement of increasing oncogene activation and over-expression with time (Benz *et al.*, 1992). It has been found that breast

cancer tumors resistant to tamoxifen retain responsiveness to pure anti-estrogens (Gottardis *et al.*, 1989; Wakeling, 1993a). Therefore, at least part of resistance to tamoxifen is related to the estrogenic activity of this antiestrogen. Treatment with EM-343 exerted a rapid inhibitory effect on both ZR-75-1 and MCF-7 human breast cancer tumors xenografted in nude mice. For up to 6 months of treatment, there was no sign of loss of response or escape from responsiveness to the inhibitory action of the anti-estrogen. Current clinical trials with EM-800, the precursor of the active antiestrogen EM-652, are showing an approximately 50% rate of response in patients treated after failure to tamoxifen (data not shown).

In summary, EM-343 shows highly potent and pure anti-estrogen activity in nude mice bearing human breast cancer xenografts. The anti-estrogen decreases both estrogen and progesterone receptor levels, this down-regulation being observed not only in the mouse uterus, but also in ZR-75-1 and MCF-7 human breast cancer tumors. The potent inhibitory effect of the anti-estrogen on the growth of both ZR-75-1 and MCF-7 human breast cancer tumors is maintained for up to 6 months, the last time interval studied. This last observation suggests that the pure anti-estrogen has the potential to improve the efficacy of endocrine therapy of human breast cancer and reduce the incidence of resistance to estrogen blockade.

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