A New Antiestrogen, 2-(4-Hydroxy-phenyl)-3-methyl-1-[4-(2-piperidin-1-yl-ethoxy)-benzyl]-1H-indol-5-ol hydrochloride (ERA-923), Inhibits the Growth of Tamoxifen-sensitive and -resistant Tumors and Is Devoid of Uterotropic Effects in Mice and Rats


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Abstract

Purpose: Tamoxifen is an antiestrogen used in women who have estrogen receptor (ER-α)-positive breast cancer. Unfortunately, resistance to tamoxifen is common in women with metastatic disease and side effects, including increased risk of endometrial cancer. Here we describe the activity of a new selective ER modulator, ERA-923, in preclinical models focused on these limitations.

Experimental Design: The ability of ERA-923, 4-OH tamoxifen, or raloxifene to inhibit estrogen-stimulated growth was evaluated in cell-based and xenograft assays with tumor cells that are sensitive or resistant to tamoxifen. Uterine effects of selective ER modulators were compared in rodents.

Results: ERA-923 potently inhibits estrogen binding to ER-α (IC_{50} 14 nM). In ER-α-positive human MCF-7 breast carcinoma cells, ERA-923 inhibits estrogen-stimulated growth (IC_{50} 0.2 nM) associated with cytostasis. In vitro, a MCF-7 variant with inherent resistance to tamoxifen (100-fold) or 4-OH tamoxifen (>1000-fold) retains complete sensitivity to ERA-923. Partial sensitivity to ERA-923 exists in MCF-7 variants that have acquired profound tamoxifen resistance. In tumor-bearing animals, ERA-923 (10 mg/kg/day given p.o.) inhibits 17β-estradiol-stimulated growth in human tumors derived from MCF-7, EnCa-101 endometrial, or BG-1 ovarian carcinoma cells, including a MCF-7 variant that is inherently resistant to tamoxifen. Raloxifene is inactive in the MCF-7 xenograft model. Unlike tamoxifen, droloxifene, or raloxifene, ERA-923 is not uterotropic in immature rats or ovariectomized mice. Consistent with this, tamoxifen, but not ERA-923, stimulates the growth of EnCa-101 tumors.

Conclusions: In preclinical models, ERA-923 has an improved efficacy and safety compared with tamoxifen. Clinical trials with ERA-923 are in progress.

Introduction

Hormonal manipulations to inhibit estrogen-stimulated tumor growth have had a major impact on the treatment of women with ER-α-positive breast cancer (1). Antiestrogen therapies include agents that competitively inhibit estrogen binding to the ER-α, such as tamoxifen, as well as agents that reduce endogenous estrogen levels, such as aromatase inhibitors or luteinizing hormone-releasing hormone agonists (2).

Tamoxifen is beneficial for the treatment of ER-α-positive metastatic breast cancer after local therapy in node-positive or node-negative breast cancer (adjuvant therapy; Refs. 3, 4) and ductal carcinoma in situ (5) as well as in breast cancer prevention in high-risk patients (6–8). However, although tamoxifen is well tolerated and has resulted in approximately 5–15% absolute reduction in recurrence and mortality (depending on the status of nodal involvement; Ref. 4), more effective treatments for ER-α-positive breast cancer are still needed. In particular, although the response rate to tamoxifen in women with metastatic disease is approximately 30% (Ref. 9; indicating that some patients have inherent resistance to the drug), it is estimated that 90% of these patients acquire resistance to tamoxifen within 1 year (10). Beyond this, side effects of tamoxifen, including endometrial cancer, gynecological symptoms, hot flushes, and vascular events such as deep vein thrombosis, can occur (6).

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2. Deceased.

3. The abbreviations used are: ER-α, estrogen receptor α; SERM, selective ER modulator; LBD, ligand binding domain; ERA-923, 2-(4-hydroxy-phenyl)-3-methyl-1-[4-(2-piperidin-1-yl-ethoxy)-benzyl]-1H-indol-5-ol hydrochloride; IMEM, improved MEM; CCS, charcoal-stripped calf serum; E2, 17β-estradiol.
These side effects are particularly problematic when tamoxifen is used in adjuvant or preventative settings.

In the last decade, it has become clear that tamoxifen and other nonsteroidal antiestrogens may be described as SERMs capable of eliciting tissue-selective effects that allow the drug to exert both estrogen antagonist and agonist properties, depending on the tissue type. In particular, tamoxifen inhibits the estrogen-induced growth of breast cancer cells, but it also has estrogentic or agonist effects that include the preservation of bone mineral density (11, 12), reductions in LDL-cholesterol in postmenopausal women (13), and uterine proliferation associated with an ~2-fold increase in endometrial cancer in one large 5-year study (14). Therefore, SERMs such as the tamoxifen analogues droloxifene (15), idoxifene (17) or those structurally related to raloxifene (18), such as LY 353381 (19) and EM-652 (20), have been developed that would either be useful in the adjuvant or prevention setting with reduced side effects compared with tamoxifen or overcome tamoxifen resistance. These nonsteroidal antiestrogens are distinct from a 7-α estradiol derivative, such as the pure antiestrogen ICI 182780 that is effective in tamoxifen-resistant tumors in the laboratory (21) and the clinic (22).

Although SERMs are structurally related, each drug has a distinct agonist and antagonist pharmacological profile (23). The basis for these differences is incompletely understood, but it can be attributed to similar but distinct conformations of ER-α induced by different SERMs (24–27), tissue-specific expression of coactivators or corepressors that bind ER-α (28), as well as the expression profile of ER-α and ER-β (29, 30). For example, E2, 4-OH tamoxifen (25), and raloxifene (24) interact with common amino acid contact residues in the LBD of ER-α. However, the unique alkylaminoethoxy side chain of both SERMs force helix 12 of the LBD, which normally acts like a trap door to seal the ligand binding pocket when bound to estradiol, to move into a position occupied by coactivators that enhance transcriptional efficacy. The exact conformational changes induced by these SERMs in the LBD, although similar, are distinct (24, 25).

We have identified a new SERM, ERA-923 (Fig. 1), that is based on the 2-phenylindole core found in zindoxifene and ZK 119101 (31, 32). Zindoxifene, which does not contain an alkylamino side chain, had estrogenciic activity on the uterus (31), and its active metabolite actually stimulated the proliferation of MCF-7 breast carcinoma cells (33). Moreover, zindoxifene was inactive in a breast cancer trial (34). When an alkylamino side chain was added to the 2-phenylindole core (ZK 119101), the molecule had reduced agonistic properties on the uterus compared with zindoxifene (32). It was reasoned that the side chain in ZK 119101 was too flexible to provide optimal displacement of helix 12 of the LBD upon binding (35). Therefore, ERA-923 was created by rigidifying the alkylamino side chain of ZK 119101. A similar moiety is found in other SERMs, such as raloxifene, LY 353381, and EM-800 (36), which have little or no uterotropic activity. ERA-923 was devoid of uterotrophic activity in immature rats compared with ZK 119101 or raloxifene (35) and, as reported here, had excellent anti-breast cancer activity in experimental models.

**MATERIALS AND METHODS**

**Cells.** Sublines of the human breast carcinoma MCF-7, designated MCF-7 SB, MCF-7 RC, and MCF-7 BK were generously provided by Drs. Susan Bates (National Cancer Institute, Bethesda, MD), Robert Clarke (Georgetown University, Washington, DC), and Benita Katzenellenbogen (University of Illinois, Urbana, IL), respectively. MCF-7 cells selected for resistance to 4-OH tamoxifen, designated LCC2 and MCF-7 TOT, were obtained from Drs. Robert Clarke and Benita Katzenellenbogen, respectively (37, 38). The human ovarian carcinoma cell line, BG-1, was generously provided by Dr. Kenneth Korach (National Institute of Environmental Sciences, Research Triangle Park, NC). All other cell lines were obtained from the American Tissue Culture Collection (Rockville, MD).

Drug-sensitive breast cancer cell lines were maintained in IMEM with zinc (Life Technologies, Inc.; Gaithersburg, MD) supplemented with 1× nonessential amino acids, 0.01 μM HEPES, 2 μg/ml insulin, 2 mg/ml glutamine, 2 mg/ml proline, 50 μg/ml gentamicin, and 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.). LCC-2 cells were maintained in IMEM supplemented with 1× nonessential amino acids, 50 μg/ml gentamicin, and 5% CCS. This media is designated LCC2. Preparation of CCS was done exactly as described (39). MCF-7 TOT cells were maintained in the same media as MCF-7 cells plus 1 μM 4-OH tamoxifen (RBI, Natick, MA).

**ER Binding Assay.** Lysates of A549 human lung carcinoma cells that were infected with an adenovirus encoding the full-length, wild-type human ER-α (40, 41) were prepared by washing cells in isotonic buffered saline and then washing once with 1 mM Tris-Cl and 1 mM EDTA (pH 7.4). After cells were scraped on ice, they were homogenized using a Brinkman Poly-
tron, and lysates were centrifuged for 1 h at 100,000 × g. The protein concentration of the resultant supernatant was determined by the Bradford method (Bio-Rad, Hercules, CA). For binding assays in 96-well dishes, 100 μl of supernatant (~1–5 μg of protein) were mixed with 10 μl of 12 nM [³H]-E₂ (70 Ci/mmol; NEN, Boston, MA) and 10 μl of ERA-923. Twelve concentrations of ERA-923 ranging from 20 pm to 10 μM were used to obtain IC₅₀ values. After incubation for 2 h at room temperature, unbound material was aspirated, the plate was washed three times with 300 μl of PBS containing 1 mM EDTA (pH 7.4), and, after aspiration, liquid scintillation cocktail (Optiphase Supermix, Wallac, Gaithersburg, MD) was added to the wells. Radioactivity was determined using a liquid scintillation counter.

Analysis of ER-α Expression and Ligand Binding in Cells. For ER-α expression, various cell lines were plated in phenol red-free media with 5–10% CCS in a six-well dish. When the cell cultures reached 90% confluence, they were extracted in 200 μl M-PER (Pierce) containing 1× protease inhibitor cocktail (PharMingen; San Diego, CA) and 0.4 M NaCl. The resultant lysates were centrifuged for 14,000 rpm for 5 min. The protein content of the resultant supernatant was determined by the Bradford method. Ten μg of protein was resolved in SDS-PAGE, transferred to polyvinylidene difluoride membranes, and the blots were quenched in 3% BSA + 3% milk in Tris-buffered saline (1 h) and then incubated overnight with 1 μg/ml mouse anti-ER-α antibody (sc-8002; Santa Cruz Biotechnology, Santa Cruz, CA) followed by a 1:1500 dilution of mouse anti-antibody (Chemicon, Temecula, CA). The blots were developed with a 1:10,000 dilution of horseradish peroxidase-antimouse IgG (Transduction Labs; Lexington, KY) with subsequent West Pico SuperSignal enhanced chemiluminescence detection according to the manufacturer directions (Pierce, Rockford, IL).

The ability of ER to bind E₂ was assessed by the adaptation of previous methods (42). Briefly, MCF-7 cells were harvested with 1 mM of EDTA prepared in Dulbecco’s PBS solution without calcium or magnesium and resuspended in IMEM containing 5% CCS. One million cells in 1 ml were incubated with 10 nM [³H]-E₂ (162 Ci/mmol; Amersham Pharmacia Biotech, Boston, MA) in the presence or absence of 1000 nM unlabeled E₂ for 30 min at 37°C. After rinsing the cells four times with 1 ml of ice cold PBS containing 1% Tween 80, cells were suspended in 200 μl PBS, transferred to scintillation vials containing 1 ml of ethanol plus 8 ml of scintillation fluid (Bio-Safe; Research Products International Corp., Mount Prospect, IL), and counted. After protein determination, the total number of specifically bound receptors was determined.

Cell Growth Assay. In experiments where estrogen-stimulated growth was examined, a flask of MCF-7 cells was placed in LCC2 media that was devoid of phenol red (designated LCC2-PR) for ~12 h. Cells were then trypsinized and plated at ~500–750 cells/well in 96-well tissue culture plates. E₂ (Sigma Chemical Co., St. Louis, MO), ERA-923, raloxifene, 4-OH tamoxifen (RBI), or ICI 182780 (obtained from AstraZeneca Pharmaceuticals; Wilmington, DE) were added to LCC2-PR media, and the cells were cultured for 5–7 days. Cell growth was assessed at the end of these time points by the tetrazolium method using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide as described previously (43). Note that the best results were achieved when cells were plated at low density. If plating density was too high, the ability of estrogen to stimulate growth was blunted.

Cell Cycle Analysis. Sixty-five thousand MCF-7 SB cells were plated in T-75 flasks and preincubated in LCC2-PR media for 12 h and then treated with the same medium with or without 0.03 nM E₂ for 3 days. Other cells were treated with E₂ plus ERA-923 or tamoxifen for 3 days. Cells were harvested by trypsinization and prepared for analysis by fluorescence-activated cell sorting using propidium iodide staining as described previously (44). Care was taken to collect both trypsinized cells and cells that may have been floating before trypsinization to insure that apoptotic cells, if present, were detected. Results from an individual experiment were confirmed in other experiments with other MCF-7 sublines.

In Vivo Experimentation with MCF-7 or BG-1 Tumors. One to 7 days before tumor implantation, athymic nu/nu female mice (Charles River Laboratories, Wilmington, MA) that were either intact or ovariectomized had pellets (Innovative Research of America, Sarasota, FL) that contained E₂ (0.36–1.7 mg/pellet; 60 or 90-day release) or a placebo implanted s.c. into the intrascapular region using a 10-gauge precision trochar. Subsequently, the animals were injected s.c. with either 1×10⁷ MCF-7 cells or BG-1 cells in the region of breast tissue. For MCF-7 and BG-1 cells, Matrigel (BD Biosciences; Bedford, MA), a basement membrane matrix preparation, was combined with tumor-cell suspensions (1:1, v/v) before injection to insure a good tumor take. In some experiments, treatment with experimental drugs began within 24 h of tumor implantation. Alternatively, tumors were allowed to attain a mass of ~200–300 mg, and then animals were randomized into treatment groups. Depending on the experiment, there were 5 or 15 animals/experimental group. Mice were treated either i.p. or p.o. with ERA-923, tamoxifen, or raloxifene prepared in 1% Tween 80 in saline. Tumor mass ([Length × Width²]/2) was determined every 3–7 days for up to 60 days. A two-tailed Student t test was used to determine statistical significance.

All animal studies were done according to established guidelines and approved by an internal animal protocol review committee.

In Vivo Experimentation with EnCa-101 Adenocarcinomas in Xenografts. EnCa-101 is a human endometrial adenocarcinoma that is continuously passaged in animals in the presence of estrogen (45). Experimentation with EnCa-101 was carried out as described previously. Briefly, tumors were maintained in castrated, athymic, BALB/c, nu/nu mice (Harlan Sprague Dawley, Indianapolis, IN). After the tumor achieved a sufficient size, it was harvested from one (anesthetized) animal and approximately three 1-mm³ pieces were transplanted into additional mice. One day later, animals were implanted with a 1.7-mg E₂ slow-release pellet or a placebo pellet (Innovative Research of America, Sarasota, FL). Four tumors were placed in each animal (two in each flank). Mice were then treated either i.p. or p.o. with ERA-923 or tamoxifen prepared in saline containing 1% Tween 80 or the vehicle alone. Drug vials were coded so the operator did not have knowledge as to which treatment was administered until the end of the experiment. Tumors were measured biweekly with Vernier calipers, and the
geometric mean diameter was determined. A two-sided t test and the error term from an analysis of covariance were used in the analysis of data.

**Evaluation of Uterine Effects in Short-Term Models.**
Ovariectomized nude mice 6 days of age or immature, intact, Sprague Dawley rats (18 days of age; H11349 35 g) were weighed before housing and distributed randomly into experimental groups. Animals received food and water ad libitum. The diet was tested to ensure that estrogens were nondetectable. The animals were housed in rooms with computer-controlled temperature and humidity with lights on a 12-h on/off cycle. In studies with nude mice where uterotrophic effects were assessed, animals were given candidate drugs p.o. for 7 days. The uterotrophic effects of drugs in immature rats were studied by injecting animals s.c. with the vehicle (50% DMSO:50% saline), 0.5 /H9262 g ethinyl estradiol, ERA-923, or raloxifene, for 3 consecutive days. On day 4 (rat) or 8 (mice), the animals were euthanized by CO2 asphyxiation, body weights were measured, and uteri were removed. The uteri were dissected free of adipose tissue, blotted free of luminal fluid, and the wet weight was determined. In some experiments, uteri were harvested from the same animals used to assess antitumor effects. The antiuterotropic effects of these agents were determined by coadministration of both agents. In rats, a uterine sample from one horn (H11011 0.5 cm) was placed in 10% buffered formalin. Fixed tissue was embedded in paraffin, stained with hematoxylin, and counterstained with eosin. The parameters scored include: epithelial hypertrophy/hyperplasia, myometrial hypertrophy, luminal distention, stromal eosinophilia, and luminal epithelial apoptosis.

**RESULTS**

**ERA-923 Competes with E2 for Binding to the ER-α.**
The ligand binding activity of ERA-923 was initially identified in a cell-free assay system. In this assay, the ability of test agents to inhibit the binding of 1 nM [3H]-E2 to cell lysates enriched with ER-α was examined. ERA-923 was a potent and specific inhibitor of E2 binding to ER-α (IC50 ,1 4 7n M ; n = 7). The compound was slightly less potent than the natural ligand, E2 (IC50 ,3 1n M ; n = 97) but was considerably more potent than tamoxifen (IC50 , 197 43 n M ; n = 3).

**Effect of Antiestrogens on Estrogen-stimulated Growth in MCF-7 Cells in Vitro.** The human breast carcinoma cell line, MCF-7, is one of the few ER-α positive tumor cell lines that maintains sensitivity to estrogen in vitro and is absolutely dependent upon estrogen for growth in nude mice (46). This cell line was fundamental in substantiating that tamoxifen would be useful in ER-positive breast cancer (47). Therefore, the activity of ERA-923 was examined in MCF-7 cells.

In vitro, estrogen stimulated the growth of MCF-7 cells provided that estrogen and phenol red (a pH indicator with a structure similar to diethylstilbestrol) were removed from the media (48). Under these experimental conditions, 0.03 nM E2 stimulated growth ~3–4-fold in MCF-7 cells compared with untreated (control) cells (Fig. 2, left panels). When coadministered with 0.03 nM E2, the IC50s of ERA-923 and 4-OH tamoxifen (the active metabolite of tamoxifen) needed to inhibit estrogen-stimulated growth were 0.21 ± 0.16 (x ± SD; n = 4 independent experiments) and 0.20 ± 0.15 nM (n = 2), respec-
tively (Fig. 2, left panels). The inhibitory effects or ERA-923 were specific because the IC$_{50}$ shifted approximately more than an order of magnitude if the amount of E$_2$ was increased 10-fold. The IC$_{50}$ values with raloxifene and tamoxifen done under the same conditions were 7.5 ± 3.5 nM (n = 2) and 500 nM (n = 1), respectively, and were consistent with previous reports (47, 48).

Whereas many drugs useful in cancer are cytotoxic, induce apoptosis, and are given on an intermittent basis, drugs such as tamoxifen or an epidermal growth factor receptor inhibitor are cytostatic: they do not cause apoptosis and are given on a daily basis (44, 49). The effects of ERA-923 on the cell cycle were studied to determine whether the drug behaved as a cytostatic or cytotoxic agent. This would help guide dosing regimens in animals. In these experiments, MCF-7 cells were first synchronized by depletion of serum and estrogen. The percentage of cells in the G$_1$-G$_0$, S, and G$_2$-M phase of the cell cycle were 86, 5, and 8%, respectively. Cells treated with E$_2$ alone entered the cell cycle, because the percentage of cells in G$_1$-G$_0$, S, and G$_2$-M were respectively 67, 19, and 13. Cells treated with E$_2$ plus 30–1000 nM ERA-923 were arrested in the cell cycle, because the percentage of cells in G$_1$-G$_0$, S, and G$_2$-M were similar to untreated cells. Similar results were obtained with E$_2$ plus 3000 nM tamoxifen. No evidence for apoptosis was observed with ERA-923. These data are similar to the findings with tamoxifen (49), are consistent with the prediction that ERA-923 is cytostatic but not cytotoxic, and suggested that chronic daily dosing would be needed to achieve activity in animals.

Specificity of ERA-923 Evaluated in ER-α-positive and ER-α-negative Cell Lines. To explore further the specificity of ERA-923, the growth inhibitory properties of the drug were compared in a panel of 19 tumor lines grown, derived from prostate, endothelial, breast, lung, colon, ovarian, and leukemic origin, in serum-containing medium. The IC$_{50}$ of ERA-923 was 92.3 ± 101 nM (x ± SD; n = 2) for MCF-7 cells. In contrast, 50–68-fold more drug was needed to achieve an IC$_{50}$ in 17 other human tumor cell lines that were ER-α-negative and in one breast carcinoma cell line (T47D) that expressed ER-α but did not respond to tamoxifen (data not shown).

Effect of ERA-923 on Tamoxifen-resistant Cell Lines in Vitro. Tamoxifen resistance occurs frequently in patients. We therefore sought to determine whether ERA-923 could inhibit the growth of tamoxifen-resistant cells. We initiated these studies by examining the effect of ERA-923 on tamoxifen-resistant MCF-7 cell lines that have either an acquired or an inherent resistance to antiestrogens.

One resistant cell line with acquired resistance, MCF-7-TOT, was established by chronic exposure of MCF-7 cells to increasing amounts of 4-OH tamoxifen up to 1 μM (Ref. 38; Fig. 2, right panels). Resistance was manifested in two ways. First, E$_2$ had almost no stimulatory effect on cell growth (and therefore, the cells were partially estrogen-independent). Second, ~10,000-fold more 4-OH tamoxifen (10 μM) was needed to inhibit the growth of resistant cells compared with the sensitive cells. This inhibition could not be competed by E$_2$ and, therefore, was not mediated by an ER-regulated pathway. Furthermore, 4-OH tamoxifen was cytotoxic only at exceptionally high concentrations that are not found in the clinical situation. In contrast, MCF-7-TOT cells were ~20-fold resistant to ERA-923, and this effect was estrogen-dependent. The response of MCF-7-TOT cells to ERA-923 was unusual, inasmuch as ERA-923 inhibited growth by only 25% at the highest concentration tested (0.3 μM), and the maximal effect reached a plateau. (Concentrations of ERA-923 >1 μM were not tested in these experiments because inhibitory effects are not estrogen-specific above this dose.) Resistance in MCF TOT cells was not explained by a reduction in the amount of the ER-α (see below). Furthermore, the ER-α seemed to be functional because the growth of MCF-7 TOT cells, although ~10,000-fold resistant to the pure antiestrogen, ICI 182,780, was inhibited ~80% by 100 nM ICI 182,780 (compare Figs. 2 and 3).

A second model that had acquired resistance to tamoxifen was examined. In this case, another MCF-7 subline was selected for resistance to 4-OH tamoxifen by chronic exposure to the drug (37). This resistant cell line, designated LCC2, responded in the same manner as the MCF-7-TOT cell line. Growth of LCC2 cells were not responsive to E$_2$, and tamoxifen resistance was complete and estrogen-independent. In contrast, resistance to ERA-923 increased only 10-fold, and the antiestrogenic effects were competed by estrogen (data not shown). As reported previously, the relative levels of ER-α were unchanged in these cell lines (37). Similar findings compared with ERA-923 were obtained with the pure antiestrogen ICI 182,780 in the LCC2 model.

These in vitro results suggest that cells selected for tamoxifen resistance maintain partial sensitivity to ERA-923. Unfortunately, because both resistant cell lines do not grow well in animals, no in vivo experimentation was possible. Therefore, other meaningful comparisons were sought by comparing the relative response of three MCF-7 cell lines to tamoxifen, 4-OH tamoxifen, and ERA-923.

It is well known that clonal variation in MCF-7 cell lines exists (50). The MCF-7 sublines examined here were obtained...
from Drs. Susan Bates (National Cancer Institute), Robert Clarke (Georgetown University), and Benita Katzenellenbogen (University of Illinois), designated MCF-7 SB, MCF-7 RC, and MCF-7 BK, respectively (Table 1). It was found that estrogen maximally stimulated growth -3.5-fold in MCF-7 BK or MCF-7 RC cells, whereas MCF-7 SB were maximally stimulated 2-fold by estrogen. Furthermore, MCF-7 SB were 10-40-fold resistant to tamoxifen and >1000-fold resistant to 4-OH tamoxifen compared with two other MCF-7 sublines. Therefore, MCF-7 SB cells were inherently resistant to tamoxifen. Nevertheless, the sublines were equally sensitive to ERA-923. These data suggest that ERA-923 can overcome inherent resistance to tamoxifen.

To further explore the basis of resistance, the expression levels of ER-α were examined by immunoblot and ligand binding assays in a variety of cell lines (Fig. 4). Although all cell MCF-7 subclones had significant levels of ER-α, the relative expression of ER-α determined by immunoblot assay among cells lines was MCF7-BK = MCF-7 TOT > MCF-7 RC > MCF-7 SB > BG-1 > BT-474. The methodology used to determine ER-α levels seems to be valid, because, consistent with a previous report, BT-474 cells had significantly less ER-α than MCF-7 cells (51). The immunoblot data in MCF-7 subclones was consistent with the ligand binding analysis inasmuch as the amount of [1H]-E2 specifically bound to MCF-7 BK, MCF-RC, and MCF-SB cells was 605 ± 85, 451 ± 9, and 258 ± 4 femol/mg (mean ± SD; n = 3), respectively. These data raise the possibility that ERA-923 is more effective than tamoxifen in MCF-7 subclones that have lower amounts of ER-α. The basis of resistance in the tamoxifen-selected cell lines remains to be determined. However, it has been reported that the MCF-7 cells selected with 4-OH have either no change (LCC-2) or a 3-fold decrease (MCF7 TOT) in E2 binding compared with parental cells (37, 38). ERA-923 Inhibits Estrogen-stimulated Tumor Growth in a Mouse Xenograft Models. The ability of ERA-923 to inhibit tumor growth was tested in mice harboring tumors derived from MCF-7 cells. In this model, MCF-7 cells require exogenous estrogen supplementation for growth in nude mice because these mice have endocrine deficiencies and do not have estrus cycles. Therefore, animals were implanted with 1.7- or 0.72-mg slow-release E2 or placebo pellets 1-7 days before the administration of MCF-7 cells. Because tamoxifen is frequently given to patients who already have a tumor burden (i.e., patients who are node-positive), experiments were initially performed on animals that already had established tumors (Fig. 5). Under these conditions, the tumor was allowed to grow for 3 months before the administration of the drug. The results show that ERA-923 inhibits tumor growth by 88%. Tumor shrinkage was not statistically different compared with MCF-7 SB by a two-tailed Student t test; *, P < 0.05; **, P < 0.01.

Table 1  Relative sensitivity of MCF-7 cell lines to estrogen and antiestrogens in vitro

<table>
<thead>
<tr>
<th>Cell linea</th>
<th>Fold-estrogen stimulation</th>
<th>IC50 (nM)b</th>
<th>IC50 (nM) tamoxifen</th>
<th>IC50 (nM) 4-OH tamoxifen</th>
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<tbody>
<tr>
<td>MCF-7 SB</td>
<td>1.93 ± 0.28 (3)</td>
<td>0.23 ± 0.16 (2)</td>
<td>10795 ± 1840 (5)</td>
<td>7448 ± 2560 (5)</td>
</tr>
<tr>
<td>MCF-7 RC</td>
<td>3.56 ± 0.57 (9)*</td>
<td>0.40 ± 0.20 (2)</td>
<td>1000 (1)</td>
<td>6.50 ± 3.5 (2)**</td>
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<tr>
<td>MCF-7 BK</td>
<td>3.48 ± 0.21 (9)**</td>
<td>0.28 ± 0.110 (2)</td>
<td>246 ± 215 (3)**</td>
<td>&lt;1.0 (n = 2)</td>
</tr>
</tbody>
</table>

a Cell line codes are: MCF-7 SB from Dr. Susan Bates; MCF-7 RC from Dr. Robert Clarke; and MCF-7 BK from Dr. Benita Katzenellenbogen.
b Fold-estrogen stimulation (versus untreated cells) and IC50 determination were determined by growth in phenol-red free, charcoal-stripped media supplemented with 0.03 nM 17β-estradiol compared with 0.03 nM 17β-estradiol plus candidate inhibitor. After 6 days exposure, cell growth was estimated by the MTT assay. Values are mean ± SE; the number of independent determinations is indicated in parenthesis.

Table 1. Relative sensitivity of MCF-7 cell lines to estrogen and antiestrogens in vitro.
observed with up to 25 mg/kg/day of ERA-923 (data not shown). The minimum effective dose needed to achieve statistically significant inhibition based on numerous experiments was determined to be 3.0 mg/kg/day (data not shown). The effects of ERA-923 on estrogen-induced growth were specific, inasmuch as 10 mg/kg/day ERA-923, given alone, did not inhibit the growth of tumors that were devoid of the ER-α (data not shown).

Because tumors were allowed to grow to a predetermined size in the MCF-7 model, this necessitated that many animals be excluded from the study because their tumors were either too small or too large and, therefore, inefficient. To expedite the experiments, studies were performed where the drug was administered to animals 1 day after tumor implantation. Similar to the established tumor model, 10 mg/kg/day ERA-923 significantly inhibited tumor growth beginning on day 14 (28% compared with the estrogen-treated group) and caused tumorstasis (37–45% inhibition) for the rest of the experiment (data not shown). Under these conditions, tumorstasis persists for at least 14 days after termination of the dosing of ERA-923, despite the fact that tumor growth continued in the animals treated with E2 alone. In contrast, administration of ERA-923 for only 2 weeks before tumor implantation was not as effective as continual administration of the drug before and during to after tumor implantation.

The effects of tamoxifen, ERA-923, and raloxifene were compared (Table 2). Two MCF-7 sublines were studied, because we knew that the MCF-7 SB subline was relatively resistant to tamoxifen compared with two other sublines (MCF-7 RC and MCF-7 BK). In tumors derived from MCF-7 SB cells, tamoxifen at doses up to 25 mg/kg (p.o.; days 1–43; qd) did not inhibit tumor growth (maximal inhibition of growth was 13% versus untreated controls), whereas 5 mg/kg and 25 mg/kg ERA-923 inhibited tumor growth 30 and 65%, respectively, when compared with the control group. The inactivity of tamoxifen was not attributed to the drug itself, because the drug inhibited an estrogen-stimulated increase in uterine wet weight in the same tumor-bearing animals (see below), and tumors derived from MCF-7 RC cells were sensitive to both tamoxifen and ERA-923 (Table 2). Raloxifene when given at 10 or 25 mg/kg/day had minimal inhibitory effects (7–12%) on the growth of tumors derived from either MCF-7 subline. These data suggest that ERA-923 may be able to inhibit the growth of tamoxifen-resistant tumors.

To insure that the effects of ERA-923 were not limited to MCF-7 tumors, additional experiments were performed with an ovarian adenocarcinoma designated BG-1 (Fig. 6) as well as the human endometrial carcinoma EnCa-101 (Fig. 7). BG-1 tumor cells express ER-α (Fig. 4) and overexpress an ER coactivator, amplified in breast cancer-1 (52). Tumors derived from BG-1 cells grew in nude mice in an estrogen-dependent fashion, because tumor growth was observed only in animals implanted with the E2 pellet. ERA-923 (20 mg/kg; qd; p.o.) was highly effective at inhibiting estrogen-stimulated growth of BG-1 tumors (Fig. 6). At the end of the experiment, tumor regression was seen in 13 of 15 animals, and no tumor was found in 2 animals. The inhibitory effect of ERA-923 persisted for >20 days after dosing; after this period, tumor growth resumed at a slow rate (data not shown).

Before the discovery that women who take tamoxifen have a 2-fold increase in the risk of developing endometrial cancer, Satyaswaroop et al. (45) and Gottardis et al. (53) demonstrated that tamoxifen, when given alone, stimulated the growth of the human endometrial cancer EnCa-101 in a xenograft mouse model. Therefore, the EnCa-101 model is useful to evaluate the estrogenic properties of SERMs on tumors. The estrogenic and antiestrogenic properties of ERA-923 and tamoxifen were compared in a blinded study using the EnCa-101 model (Fig. 7). Animals were implanted with EnCa-101, and test agents were given daily at 0 (vehicle), 2, or 20 mg/kg p.o. to animals harboring either an E2 pellet or a placebo pellet. Results with only the 0- or 20-mg/kg tests groups are shown in Fig. 7. After a 2-week dormancy, tumor growth occurred in response to estrogen. Small but significant growth occurred in tumors given placebo estrogen pellets (vehicle). As expected, 20 mg/kg tamoxifen, when given alone, significantly stimulated tumor growth (P < 0.003). Similar but attenuated results were also observed if animals were given 2 mg/kg tamoxifen (data not shown). In contrast, 20 mg/kg ERA-923 had no significant agonistic activity (P > 0.05 versus vehicle control group) and was antiestrogenic. Remarkably, in animals given 20 mg/kg ERA-923 plus estrogen, tumors regressed or disappeared such that only one of five animals had tumors at the termination of the experiment. In animals given 2 mg/kg ERA-923, no agonistic activity was observed, whereas estrogen-stimulated tumor growth was not significantly inhibited.

### Table 2 Tamoxifen inhibits the growth of tumors derived from MCF-7 RC but not MCF-7 SB cells

<table>
<thead>
<tr>
<th>Treatment vs. control (%)</th>
<th>MCF-7 SB</th>
<th>MCF-7 RC</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2 (0.72-mg pellet)</td>
<td>100 ± 10.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100 ± 11.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>E2 + ERA-923</td>
<td>70.2 ± 9.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>46.4 ± 5.3&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>5 mg/kg/day (p.o.)</td>
<td>47.4 ± 11.6&lt;sup&gt;**&lt;/sup&gt;</td>
<td>46.8 ± 6.9&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 mg/kg/day (p.o.)</td>
<td>34.9 ± 3.7&lt;sup&gt;***&lt;/sup&gt;</td>
<td>25 mg/kg/day (p.o.)</td>
</tr>
<tr>
<td>20 mg/kg/day (p.o.)</td>
<td>9.7* 7.0&lt;sup&gt;**&lt;/sup&gt;</td>
<td>20 mg/kg/day (p.o.)</td>
</tr>
<tr>
<td>25 mg/kg/day (p.o.)</td>
<td>15.0</td>
<td>25 mg/kg/day (p.o.)</td>
</tr>
<tr>
<td>E2 + raloxifene</td>
<td>78.4 ± 7.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.4 ± 26.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 mg/kg/day (p.o.)</td>
<td>93.7 ± 13.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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</table>

<sup>a</sup> Animals implanted with estrogen pellets were treated with ERA-923, tamoxifen, or raloxifene at the indicated doses 1 day after injection of tumor cells. Antiestrogens were given once daily. Tumor measurements were determined every week. The effects at the termination of the experiment, on days 43 or 44, are reported. The effect of the treatment versus the control was calculated by dividing the average tumor size in animals that received E2 plus the candidate antiestrogen by the E2-treated group and then multiplying by 100. Animals implanted with MCF-7 SB and MCF-7 RK cells and treated with E2 had tumors ~1000 and 800 mg, respectively.

<sup>b</sup> Values are X ± SE; n = 9–20 mice except in the E2 treated group, which was 30.

<sup>c</sup> *, P < 0.05; **, P < 0.01; ***, P < 0.001 by two-tailed Student’s t test versus estrogen-treated group.
Effect of ERA-923 on Uterine Tissue in Athymic Mice.

Tamoxifen and droloxifene have anti-breast cancer activity and increase uterine wet weight in mice and immature rats (16, 54). Because the uterotropism of tamoxifen may be more profound in mice than in rats (55), comparisons between ERA-923, tamoxifen, and droloxifene were undertaken initially in nude mice. Animals were given candidate agents p.o. once per day for 7 days. Uteri were removed 24 h after the last dose, and the wet weights of the uteri were determined. It was found that 10 mg/kg ERA-923 had no effect compared with untreated animals or animals given the vehicle as a control, whereas both tamoxifen and droloxifene were uterotropic (Table 3). A dose-response relationship on uterine wet weight was established with tamoxifen, whereas ERA-923 was not effective at all doses tested (0.3–10 mg/kg/day; data not shown). Consistent with these results, 10 mg/kg ERA-923 did not increase uterine wet weight in nude mice if the drug was given daily for 28 or 44 days (i.p. or p.o. dosing, respectively), whereas 10 mg/kg tamoxifen (p.o. dosing; 44 days) increased uterine wet weight 3-fold (data not shown).

The antiuterotropic effect of ERA-923 was evaluated in the same (tumor-bearing) nude mice used to determine the antitumor effects. In these experiments, the uterine wet weight was examined after animals were given estrogen pellets with or without ERA-923 or tamoxifen (Table 4). In these studies, E2 treatment led to a 4-fold increase in uterine wet weight. This effect was inhibited in a dose-dependent manner when treatment was combined with ERA-923. In general, when dramatic an-

![Fig. 6](image69x326 to 286x521)

**Fig. 6** Effect of ERA-923 on ER-α positive BG-1 human ovarian tumors. Nude mice implanted with 0.72-mg E2 (E2) 60-day slow-release pellets or placebo pellets (no E2 group) were inoculated with 1 x 10^7 BG-1 cells and treated daily with 20 mg/kg/day ERA-923 (p.o., qd). For animals treated with E2 or placebo pellets, tumors were allowed to reach 200–300 or 100 mg, respectively, before treatment with ERA-923. *P < 0.05; **P < 0.001.

![Fig. 7](image242x551 to 543x692)

**Fig. 7** Effect of ERA-923 and tamoxifen on a human endometrial adenocarcinoma, EnCa-101. Nude mice implanted with explants of EnCa101 were given 1.7-mg 60-day slow-release E2 pellets (E2, ▲) or placebo pellets (vehicle, ○). One day after tumor implantation, animals with or without E2 pellets were treated with 20 mg/kg ERA-923 (▲ and △, respectively). Alternatively, animals with placebo pellets were treated with 20 mg/kg/day tamoxifen (○; p.o., qd). Tumor size was measured weekly. Values shown are mean ± SE; n = 5–7 animals/group. Asterisks, the treatment group was significantly different from the appropriate control (vehicle or estrogen-treated group); P < 0.05 using a two-tailed t test and the error term from an analysis of covariance.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Uterotropic evaluation of ERA-923 in nude micea</th>
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</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Uterine wet weight (mg)b</td>
</tr>
<tr>
<td>Control (vehicle alone)</td>
<td>28.0 ± 3.2</td>
</tr>
<tr>
<td>Control + 10 mg/kg ERA-923</td>
<td>32.4 ± 6.0</td>
</tr>
<tr>
<td>Control + 10 mg/kg tamoxifen</td>
<td>97.0 ± 6.2*</td>
</tr>
<tr>
<td>Control + 10 mg/kg droloxifene</td>
<td>71.2 ± 5.6*</td>
</tr>
</tbody>
</table>

a Ovariectomized mice were treated with the test agent (p.o.) qd for 7 days.

b Values are x ± SE; n = 5–19 animals/group.

**Table 4** Antiuterotropic evaluation of ERA-923 in nude micea

| Treatment | Uterine wet weight (mg)b |
| Control (placebo pellet) | 53.8 ± 9.8 |
| E2 pellet (0.72 mg) | 220.6 ± 8.8 |
| E2 pellet + 0.3 mg/kg/day ERA-923 | 174.0 ± 12.1* |
| E2 pellet + 1.0 mg/kg/day ERA-923 | 149.0 ± 22.3* |
| E2 pellet + 3.0 mg/kg/day ERA-923 | 82.2 ± 3.8* |
| E2 pellet + 10 mg/kg ERA-923 | 61.1 ± 6.4** |
| E2 pellet + 10 mg/kg/day tamoxifen | 113.6 ± 17.24** |
| E2 pellet + 25 mg/kg/day tamoxifen | 97.0 ± 13.1** |

a Ovariectomized nude mice implanted with placebo or 0.72-mg E2 pellets were given candidate antiestrogens (p.o.) qd for ~40 days.

b Values are x ± SE; n = 5–7 animals/group.

* P < 0.05; ** P < 0.01; two-tailed Student t test versus vehicle alone.
tiuterotropnic effects were observed with ERA-923, the compound also had antitumor activity (compare with Fig. 6). Tamoxifen was also anttiuterotropnic in this assay.

The effects of ERA-923 and raloxifene on the uterus were evaluated further in immature rats. In these studies, 0.5 μg/rat ethinyl estradiol (~14 μg/kg) increased uterine wet weight ~300% of control values. Ten or 100 μg/rat ERA-923 (approximately 0.2 and 2.0 μg/kg) either did not increase or slightly decreased uterine wet weight versus control-treated animals. In contrast, 10 and 100 μg/rat raloxifene increased uterine wet weight 172 and 145% of control values. Hypertrophy and hyperplasia of the endometrium were associated with the effects of raloxifene. Consistent with previous reports, raloxifene induced uterotrophic effects that were blocked by antiestrogens (56) because 10–100 μg/rat ERA-923 completely blocked the raloxifene-induced uterotrophic effects.

**DISCUSSION**

The data presented here demonstrate that ERA-923 is a SERM with a distinct profile compared with tamoxifen. In particular, unlike tamoxifen, ERA-923 is devoid of uterotrophic activity and does not stimulate the growth of endometrial tumors in the EnCa-101 experimental model. These data may indicate that, in patients, ERA-923 will not increase the incidence of endometrial hyperplasia or cancer. This is in contrast to tamoxifen, where a 10- and 2-fold increase in endometrial hyperplasia and cancer has been reported, respectively (2, 57). Beyond this, ERA-923 inhibits estrogen-stimulated ER-α-dependent tumor growth with equal effects compared with tamoxifen in models sensitive to tamoxifen. In addition, ERA-923 partially or completely overcomes tamoxifen resistance (depending upon the experimental model). Therefore, ERA-923 may have utility in women who have ER-α positive breast cancer that fails to respond to tamoxifen.

The ability of ERA-923 to overcome tamoxifen resistance in experimental models raises important questions including: (a) what is the mechanistic basis of tamoxifen resistance in the clinic? (b) how well do animal models mimic the clinical phenomena? and (c) how predictive are the findings with ERA-923 for women who have failed tamoxifen therapy? The basis of resistance to tamoxifen, at both the clinical and experimental levels is complex and poorly understood (10, 58, 59). In patients who are tamoxifen-resistant, mutation or altered expression in ER-α is rare (10), and altered tamoxifen metabolism cannot account for resistance. Both experimental and clinical data suggest that a switch from tamoxifen’s antiestrogenic to estrogenic effect is associated with resistance. Consistent with this hypothesis, tamoxifen actually stimulates MCF-7 tumor growth after extended treatment (60). A similar phenomenon has been inferred from tamoxifen-resistant patients where tumor regression occurs after the termination of tamoxifen therapy (22). In one tamoxifen-stimulated experimental model, a point mutation in ER-α occurs (61) that (a) increases the estrogenicity of a tamoxifen analogue, 4-OH tamoxifen, in cells transfected with the mutant gene (62, 63); (b) causes a conversion of raloxifene from an antiestrogen to a partial estrogen (64); and (c) influences coactivator interaction with the LBD of ER-α (24). Because 4-OH tamoxifen and raloxifene induce similar but distinct con-
formations of the ligand binding domain of ER-α (24, 25), resistance to one SERM mediated by altered ER:coactivator interaction may not indicate cross-resistance to other SERMs such as ERA-923. Besides changes in the ER-α, alteration in other growth factor pathways, coactivators or corepressors, as well ER-β may contribute to resistance (59).

In experimental models, partial sensitivity to ERA-923 was observed in two cell lines selected for high-levels of resistance to the potent tamoxifen metabolite, 4-OH tamoxifen. Although these effects are modest, because resistance to tamoxifen in these models probably far exceeds the levels of tamoxifen resistance observed in the clinic, the clinical significance of the data are questionable. In contrast, ERA-923 can completely overcome tamoxifen resistance in a MCF-7 subline, designated MCF-7 SB, in both in vitro and in vivo assays. MCF-7 SB cells are inherently resistant to tamoxifen. Presumably, MCF-7 SB, which have been maintained in tissue culture for many years, are a subclone of the original MCF-7 cell line. It has been well documented that subclones of MCF-7 cells have different sensitivities to estrogen (50). The clinical relevance of the data with the MCF-7 subclones is not clear. However, because MCF-7 SB cells are ~10-fold less resistant to tamoxifen and 4-OH tamoxifen compared with the tamoxifen-selected resistant cells, they may reflect a more clinically relevant model. Additional evaluation of ERA-923 in other tamoxifen-resistant models, particularly those where tamoxifen resistance has been established in vivo (65), is warranted. Beyond this, comparative analysis of MCF-7 cell sublines may help define further the molecular basis of tamoxifen resistance. Our data raises the possibility that in these cell lines, reduction in ER-α levels or the ability of the ER-α to bind estrogen is correlated with resistance to tamoxifen whereas sensitivity to ERA-923 is retained.

The lack of agonistic activity of ERA-923 in the EnCa-101 cancer model is consistent with the lack of effect of the drug on the uterus that has been observed in rats and mice. The EnCa-101 model was validated, because tamoxifen stimulated the growth of EnCa-101 as reported previously (45, 53). If ERA-923 does not increase the incidence of endometrial cancer and uterine proliferation in humans, then it may be an improvement over tamoxifen, particularly where the drug would be given to women who have low-grade disease (i.e., in situ ductal carcinoma) or who are at high risk for developing breast cancer (i.e., the preventative setting). The profile of ERA-923 is similar to other novel nonsteroidal antiestrogens, EM-800 and its active metabolite EM-652, which are devoid of estrogenic properties in the immature rat uterus but have good antiestrogenic activity in breast cancer models (20).

Our studies suggest that raloxifene had low antiestrogenic activity in tumors derived from MCF-7. This was surprising, because raloxifene had excellent potency (equivalent to ERA-923) in the MCF-7 cell growth assays. No studies with raloxifene in the MCF-7 tumor model have been reported, although moderate antitumor activity has been reported in the N-methyl-N-nitrosourea-induced rat mammary carcinoma model as well as in the EnCa-101 model (66). It is unlikely that poor bioavailability of raloxifene can completely account for the lack of activity of raloxifene in the tumor models reported here, because we and others (67) have observed that raloxifene inhibited estrogen-stimulated uterine growth. In addition, we found that
raloxifene did not have anticancer activity if administered in a different vehicle (cyclodextrin) or if the compound was administered i.p. (and therefore, compound absorption was less of a factor). It may be that the insufficient activity of raloxifene or its metabolites at the tumor site prohibits good anti-breast cancer activity of this compound in these model systems. Alternatively, ERA-923 and raloxifene may interact with the ER-α in a distinct manner.

To determine whether ERA-923 would be useful in cancer patients in the adjuvant or preventative setting, an ideal agent should have anticancer activity as well as preserve bone density, lower cholesterol, and reduce the incidence of hot flashes. ERA-923 as well as a highly related analogue known as TSE-424 preserved bone density in rat experimental models. Therefore, similar to raloxifene, ERA-923 may be useful in women who are at risk for, or who have developed, osteoporosis, and it also may reduce the incidence of breast cancer and diseases related to high cholesterol (i.e., stroke or heart disease). Because ERA-923 does not have estrogenic effects on tumor growth in the experimental models reported here, yet can preserve bone and is a potent inhibitor of estrogen-stimulate tumor growth, ERA-923 may have some advantages over the current estrogen-replacement therapies or therapy with raloxifene for the treatment of osteoporosis.

In conclusion, we have demonstrated that ERA-923 is a novel SERM that, in preclinical models, has a superior profile compared with tamoxifen. On the basis of preclinical safety evaluation as well as Phase I clinical assessment in normal volunteers (69), ERA-923 is currently being evaluated in a Phase II trial in women with metastatic breast cancer who have failed on tamoxifen therapy.

ACKNOWLEDGMENTS
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3176 Novel ER-α Antagonist for Breast Cancer


A New Antiestrogen, 2-(4-Hydroxy-phenyl)-3-methyl-1-[4-(2-piperidin-1-yl-ethoxy)-benzyl]-1H-indol-5-ol hydrochloride (ERA-923), Inhibits the Growth of Tamoxifen-sensitive and -resistant Tumors and Is Devoid of Uterotropic Effects in Mice and Rats


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