Characterization of New Estrogen Receptor Destabilizing Compounds: Effects on Estrogen-Sensitive and Tamoxifen-Resistant Breast Cancer

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Background: Antiestrogens of the selective estrogen receptor modulator (SERM) type, such as tamoxifen, have two major limitations: their mixed agonist and antagonist profile and the development of tumor resistance. We characterized two new pure antiestrogens—ZK-703 and ZK-253—that belong to the class of specific estrogen receptor destabilizers (SERDs), which includes fulvestrant, and compared their activity with that of fulvestrant and tamoxifen. Methods: Effects of antiestrogens on the growth of estrogen-dependent breast tumors in vivo were determined using several mouse xenograft models (including the tamoxifen-sensitive tumors MCF7, T47D, and MDA366 and the tamoxifen-resistant tumors ZR75-1 and MCF7/TAM) and chemically induced (nitrosomethyl urea [NMU] and dimethylbenzanthracene [DMBA]) rat breast cancer models (groups of 10 animals). We determined the initial response and effects on hormone receptor levels and the time to relapse after treatment (i.e., time to reach a predetermined tumor size threshold). Estrogen receptor (ER) levels were determined by immunoassay. Results: ZK-703 (administered subcutaneously) and ZK-253 (administered orally) were more effective than tamoxifen or fulvestrant at inhibiting the growth of ER-positive breast cancer in all xenograft models. For example, MCF7 tumors relapsed (i.e., reached the size threshold) in 10 weeks in mice treated with tamoxifen but in 30 weeks in mice treated with ZK-703. ZK-703 and ZK-253 also prevented further tumor progression in tamoxifen-resistant breast cancer models to a similar extent (more than 30 weeks in mice with ZR75-1 and MCF7/TAM tumors). In the chemically induced rat breast cancer models, orally administered ZK-703 and ZK-253 caused a nearly complete (>80%) inhibition of tumor growth. ER levels were dramatically reduced in MCF7 tumors after 5 weeks of ZK-703 treatment compared with ER levels in vehicle-treated tumors; by contrast, ER levels in tamoxifen-treated tumors were higher than those in control tumors. Conclusion: ZK-703 and ZK-253 are potent, long-term inhibitors of growth in both tamoxifen-sensitive and tamoxifen-resistant breast cancer models. [J Natl Cancer Inst 2004;96:210–18]

The introduction of the antiestrogen tamoxifen has changed treatment of all stages of breast cancer (1). It is the method of choice not only for the treatment of advanced disease in pre- and postmenopausal women but also for prevention in women at high risk of developing breast cancer (2). Although it is effective, tamoxifen has an important drawback: the limited period of activity before resistance develops. Despite remarkable improvements in treatment options, development of endocrine resistance is one reason that breast cancer is the second most frequent cause of cancer death in women.

Tamoxifen has both estrogen agonist and antagonist activities. Resistance to tamoxifen is a complex phenomenon (3,4), although there is evidence that relapse under tamoxifen therapy is linked to the estrogenicity of the drug. Prolonged treatment with tamoxifen is associated with an increased risk for the development of endometrial cancer, which may be related to the pronounced estrogen agonistic activity of the drug, indicated by increased endometrial thickness, effects on the liver, decreased gonadotropin levels, and hot flushes (5). In the context of a treatment whose purpose is to achieve maximum estrogen deprivation, partial estrogen agonistic activity is obviously undesirable. As long as the estrogen receptor (ER) is present, transcription of estrogen-responsive genes (and tumor growth) may still be stimulated by small amounts of estrogens or antiestrogens or may even occur in a ligand-independent fashion by growth factor–mediated ER phosphorylation (6,7).

Consequently, efforts have been made to develop new antiestrogens that do not display agonist activity or lead to the development of resistance. Wakeling et al. (8) introduced the first prototype of so-called pure antiestrogens (fulvestrant, or ZM 182780) in 1991. This compound lacks estrogen agonistic activity and leads to a rapid reduction in ER levels. Because such compounds have no agonistic activity but instead destabilize the ER, resulting in complete disruption of ER-mediated growth stimulation, they appear to be an effective approach to avoiding the development of antiestrogen resistance. In phase III clinical trials, fulvestrant has proved to be as effective as the aromatase inhibitor anastrozole in patients who relapsed during tamoxifen treatment (9). Fulvestrant has very low bioavailability when administered orally, however, and must be given by intramuscular injection.

We have therefore focused our drug-finding activities on identifying a pure but orally available antiestrogen. We have synthesized a number of pure antiestrogens and characterized their structure–activity relations (10). In this article, we present the preclinical characterization of the two most potent of these compounds, ZK-703 and ZK-253, in breast cancer models. ZK-703 and ZK-253 are pure estrogen antagonists that cause effi-
cient destabilization of the ER protein in T47D breast cancer cells. The IC\textsubscript{50} (the concentration that causes 50% inhibition of ER expression) for ZK-703 is 1.29 × 10^{-10} M, in contrast with 3.32 × 10^{-10} M for fulvestrant (Parczyk K: unpublished data). Our goal was to use experimental breast cancer models to demonstrate \textit{in vivo} the relevance of ER destabilization and resistance modulation and thereby provide a reliable rationale for the translational step from the laboratory into the clinic (11).

**MATERIALS AND METHODS**

The antiestrogens 4-hydroxytamoxifen (4-OH-TAM), tamoxifen, raloxifene, EM-800, fulvestrant (ZM 182780), ZK-703, and ZK-253 (Fig. 1) and estradiol (E\textsubscript{2}) were synthesized in the laboratories of Schering AG (Berlin, Germany). The compounds were solubilized in ethanol (at 10\textsuperscript{-3} M) and stored at –20 °C. They were always diluted immediately before use to the desired concentrations in 0.01 M phosphate-buffered saline (PBS; 0.138 M NaCl, 0.0027 M KCl, pH 7.4). For \textit{in vivo} experiments, all drugs were dissolved in ethanol/arachis oil (1:9).

**Cell Lines and Culture Conditions**

The MCF7 human mammary carcinoma cell line (12) was obtained from Dr. Marc Lippman, University of Michigan. The ZR75-1, BT474, and T47D breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). All cells were routinely grown in phenol red-free RPMI-1640 medium supplemented with 10% fetal calf serum, insulin at 200 mU/mL, and 10\textsuperscript{-10} M estradiol. When the cells reached 60%–70% confluence, they were harvested using PBS containing 0.05% trypsin plus 0.02% EDTA. Cells were stored in frozen stocks in liquid nitrogen and were used for no more than 10 passages. Before each experiment, cells were grown for 3 days in phenol red–free RPMI-1640 medium supplemented with 10% charcoal-treated fetal calf serum (CCS; without estradiol) to deplete steroids.

**Cell Growth Studies**

Tumor cells were seeded in 200 μL at 5000 cells per well in 96-well plates. Cells were allowed to adhere for 24 hours, and then fresh medium (10% CCS), either with or without estradiol or antiestrogens, was added at doses ranging from 10\textsuperscript{-10} to 10\textsuperscript{-6} M. The medium was replaced after 3 days. On day 7, cells were fixed with glutaraldehyde and stained with crystal violet, and absorbance at 595 nm was recorded (13). Absorbance values were normalized to the absorbance of vehicle-treated cells, and IC\textsubscript{50} values were calculated. Each cell growth experiment were performed at least twice, and data are presented as means of the IC\textsubscript{50} values with 95% confidence intervals (CIs).

**Mouse Xenograft Breast Cancer Models**

Female NMRI athymic nude mice with a body weight of approximately 20 g (Schering AG, animal facilities) were fed with Altromin R (Altromin, Lage, Germany) diets, and autoclaved water was provided \textit{ad libitum}. Mice were caged in Macrolon type III wire mesh–bottom cages (10 mice per cage) under low-germ conditions at room temperature (22 °C) and 50%–60% relative humidity, with a daily light cycle of 10 hours dark/14 hours light. The mice were implanted subcutaneously with slow-release estradiol pellets (1.7 mg, 60-day release; Innovative Research of America, Sarasota, FL) 3 days before tumor transplantation.

MV3366 breast cancer tissue samples (14) were obtained from Helga Naundorf (Max-Delbrück-Center for Molecular Medicine [MDC], Berlin-Buch, Germany). Small tumor pieces (2 × 2 mm) were serially transplanted into nude mice. MCF7, T47D, BT474, and ZR75-1 tumor cells grown in cell culture (1.5 × 10\textsuperscript{6} cells) were suspended in Matrigel (Becton Dickinson, Heidelberg, Germany) and transplanted by a single subcutaneous injection into the left flank of nude mice. The MCF7 xenograft tumors were found to be ER-positive and progesterone receptor (PR)–positive, and their growth was strictly estrogen dependent (Hoffmann J, Schneider MR: unpublished data). The resistant MCF7/TAM subline was created by long-term treatment of MCF7 xenograft–bearing mice with tamoxifen; after development of resistance, the line was maintained by serial passaging of small tumor pieces (2 × 2 mm) in tamoxifen-treated mice.

After tumors were established (i.e., after they reached approximately 25 mm\textsuperscript{2}), mice were randomly assigned to two control groups (continue treatment with estradiol or undergo ovariectomy without estradiol) and to treatment groups that were treated daily with the antiestrogens (10 mice in all groups treated orally or subcutaneously, depending on the compound, with doses of 3–50 mg/kg). Tumor growth was assessed by determining tumor area (product of the longest diameter and its perpendicular) with calipers weekly. Experiments were designed to determine either the time to relapse after treatment or the tumor response and effects on hormone receptor levels during a certain time. Control and treatment groups were removed from the experiment when, after progression, tumor size reached a...
median value of at least 100–150 mm² (or when even larger tumors were observed in one or more mice). The data are presented as group tumor growth curves showing medians and interquartile ranges for each observation time point. If tumors (only MCF7/TAM, shown in Fig. 4) were transplanted on both flanks, both tumors were analyzed.

**Chemically Induced Rat Breast Cancer Models**

Female Sprague-Dawley rats (Tierzucht Schönwalde, Schönwalde, Germany) were caged in Macrolon type III wire mesh–bottom cages (two animals per cage) under conventional conditions at room temperature (22 °C) and 50%–60% relative humidity, with a light cycle of 10 hours dark/14 hours light. Rats were fed Altromin R and autoclaved tap water (ad libitum 24 hours/day). Breast tumors were induced in 50- to 55-day-old rats by either a single intravenous injection of nitrosomethyl urea (NMU; 50 mg/kg) or a single oral application of dimethylbenzanthracene (DMBA; 10 mg).

Rats with at least one established tumor of 150 mm² or larger were randomly assigned to four or five groups of 10 rats each to achieve an approximately equal distribution of tumors of different latencies, numbers of tumors, and total tumor area among the experimental groups. Antiestrogen treatment with ZK-703 (1 or 3 mg/kg, orally), ZK-253 (1 or 3 mg/kg, orally), or fulvestrant (3 or 10 mg/kg, orally) was started after randomization and continued for 4 weeks. Tumor area was measured weekly with calipers and was calculated as the product of the longest diameter and its perpendicular. The total area of all tumors in each animal was defined as 100% at the start of treatment, and changes in tumor area were determined relative to this value. After 4 weeks, the experiment was terminated, and tumors were excised and weighed. Data are presented as mean tumor weight per group with 95% CIs. The mean tumor weight per group in treatment groups compared with that in control (vehicle-treated) groups was used to calculate the percentage growth inhibition. The Kruskal–Wallis test was used for statistical analysis of inter-group differences.

All animal experiments were performed in accordance with national guidelines (German Animal Protection Law).

**ER and PR Assays**

At the end of the experiments shown in Figs. 2 and 6, tumors were collected and snap frozen in liquid nitrogen. For ER and PR assays, 100 mg of frozen tissue was pulverized, recovered in 0.5 mL of high-salt buffer (PBS with 400 mM KCl), vortexed, and ultracentrifuged at 100 000 × g at 0 °C. The supernatant was used for ER and PR enzyme immunoassays, according to the manufacturer’s instructions (Abbott, Wiesbaden, Germany).

Enough material was available to carry out each assay only once. Because some antiestrogens inhibited tumor growth so strongly, in some cases two or three tumors from a treatment group had to be pooled to have enough tissue for the assay.

**Drug Serum Concentration Analysis**

Serum concentrations of ZK-703 and fulvestrant were determined in mice bearing MCF7 xenograft tumors. Mice (groups of six) were injected subcutaneously with ZK-703 or fulvestrant at 10 mg/kg daily, and serum samples were obtained from three mice (chosen at random) 4 and 24 hours after the injections on certain days. Samples from the three mice from each group and time point were combined for liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analysis by internal standardization. For ZK-703, 2 mL of bovine serum albumin buffer (0.1%) was added to 100 μL of pooled serum samples, followed by vortexing and ultrasonication. For fulvestrant, 0.3 mL of bovine serum albumin buffer (pH 7) was added to 100 μL of serum and extracted with a diethyl ether–hexane mixture (1:1, vol/vol). Samples were transferred onto a solid-phase extraction cartridge, eluted with methanol, dried, and resolved in acetonitrile/water (1:1). An aliquot of 2 μL was injected into a PerkinElmer Series 200/PE Sciex-API LC/MS/MS system (PerkinElmer, Weiterstadt, Germany). Six calibration standards were prepared from three stock solutions by spiking defined amounts of ZK-703 and fulvestrant into serum in the concentration range (1–200 ng/mL). Quality-control levels in the same concentration range were also prepared. All samples per analyte were measured at the same time.
Statistical Analyses

The data for mouse tumor growth are presented as group tumor growth curves showing medians and interquartile ranges for each observation timepoint. The data were analyzed primarily by taking the time structure of the repeatedly observed tumor growth into account. Therefore, time-to-event (commonly referred to as survival) analyses were performed. An event was defined as the time at which an animal’s tumor reached a preset threshold size. Because we were interested in the time to tumor doubling, and because treatment was started when average tumor sizes were 25 mm², we set the threshold at 60 mm² (i.e., slightly above a doubled size). We also repeated all analyses using different thresholds (i.e., 40 or 80 mm²). Obtained times-to-event were compared between treatment groups; we included animals whose tumor size never reached the threshold. The log-rank test was used to determine the statistical significance of differences in tumor growth; a $P$ value of less than 0.05 was considered statistically significant.

RESULTS

In Vitro Antiproliferative Activities

We first investigated the effects of estradiol and the antiestrogens tamoxifen, fulvestrant, ZK-703, and ZK-253 on proliferation of MCF7 and T47D cells, both of which express ER and PR (15). Following steroid depletion, cells were allowed to grow for 7 days in the presence of fresh medium plus the appropriate compound before growth was measured by absorbance. In experiments in which cells were treated with estradiol plus the antiestrogens ZK-703, ZK-253, tamoxifen, or fulvestrant at doses ranging from 0.1 nM to 1 μM, the estradiol-stimulated growth of MCF7 and T47D cells was reduced in a dose-dependent manner. The IC$_{50}$ values for the antiproliferative effects of the antiestrogens are given in Table 1. Cells were also cultivated without estradiol and with increasing concentrations of antiestrogens. When used at concentrations above $3 \times 10^{-9}$ M, the partial estrogen agonist tamoxifen did not stimulate growth relative to cells growing without estrogens, whereas when the pure estrogen antagonists ZK-703, ZK-253, and fulvestrant were used at concentrations above $3 \times 10^{-8}$ M, they suppressed growth of both cell lines below the levels of cells growing without estrogens (data not shown).

Serum Estrogen and Drug Levels in the MCF7 Human Breast Cancer Xenograft Model

We next examined the effect of antiestrogens on the strongly estrogen-dependent MCF7 human breast cancer model in vivo.

Table 1. IC$_{50}$ values (the concentrations that cause 50% inhibition of growth) for the antiproliferative effect of antiestrogens on estradiol-stimulated human breast cancer cell lines

<table>
<thead>
<tr>
<th>Compound</th>
<th>MCF7</th>
<th>T47D</th>
</tr>
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<tbody>
<tr>
<td>ZK-703</td>
<td>2.4 (1.8 to 3.0)</td>
<td>2.1 (1.5 to 2.7)</td>
</tr>
<tr>
<td>ZK-253</td>
<td>1.9 (0.7 to 3.1)</td>
<td>1.3 (0.0 to 2.6)</td>
</tr>
<tr>
<td>Fulvestrant</td>
<td>3.9 (2.4 to 5.4)</td>
<td>2.2 (1.1 to 3.3)</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>45.5 (14.1 to 76.9)</td>
<td>11.8 (4.6 to 19.0)</td>
</tr>
</tbody>
</table>

In preliminary experiments (Schneider MR: data not shown), we found that supplementation with 0.36 or 1.7 mg estradiol pellets produced comparable stimulation of tumor growth in MCF7 xenograft mice. Corresponding serum estradiol levels were 0.99 nM (SD = ±0.19 nM) and 3.99 nM (SD = ±1.2 nM). In mice with no estradiol pellet supplementation, no tumor growth was observed over more than 10 weeks, demonstrating that endogenous serum estradiol levels in these mice (0.07 nM (SD = ±0.04 nM)) are too low to support tumor proliferation and also revealing the strong hormone dependence of MCF7 cells.

We then determined the optimal dose regimen of one of the new antiestrogens, ZK-703, for use in further preclinical studies. We observed a dose-dependent inhibition of tumor growth with daily subcutaneous application of ZK-703 at doses ranging from 1 to 10 mg/kg (Hoffmann J, Schneider MR: data not shown). Daily subcutaneous administration of ZK-703 and fulvestrant at equal doses (10 mg/kg) led to similar 4-hour serum levels of these drugs (Table 2). Although there were exceptions at days 8 and 15, in general, the trough-level (i.e., 24-hour) concentrations for ZK-703 were much lower than the levels 4 hours after the dose, whereas fulvestrant remained at a higher level.

Tumor Growth in ZK-703-Treated MCF7 and ZR75-1 Xenograft Mice

We next used the MCF7 breast cancer model to characterize the effect of ZK-703 on tumor growth and ER and PR expression levels. Tumor growth in this experiment (as in all of our experiments using human breast cancer models xenotransplanted into nude mice) was statistically analyzed by determining the time for tumors to reach 60 mm$^2$ (i.e., slightly above twice the average starting tumor size of 25 mm$^2$). ZK-703 treatment had a stronger effect on tumor growth than tamoxifen or fulvestrant—about as strong as ovariectomy (Fig. 2, A). The differences were statistically significant ($P<0.001$). (Similar results were obtained when the threshold was changed to either 40 or 80 mm$^2$; data not shown.) The experiment was terminated after 5 weeks, even for mice whose tumors had not reached 60 mm$^2$, and tumor ER and PR expression were analyzed. Tumors of mice treated with tamoxifen or ovariectomy had higher levels of ER expression and lower levels of PR expression than tumors of vehicle-treated mice, whereas tumors of mice treated with either fulvestrant or ZK-703 had very low levels of both ER and PR expression (Fig. 2, B). Because several samples from the treatment groups were pooled, no statistical analysis was performed.

Table 2. Mean serum concentrations of ZK-703 and fulvestrant in mice bearing MCF-7 xenograft tumors

<table>
<thead>
<tr>
<th>Day of serum sampling</th>
<th>ZK-703, ng/mL</th>
<th>Fulvestrant, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4-h sample</td>
<td>24-h sample</td>
</tr>
<tr>
<td>Day of serum sampling</td>
<td>4-h sample</td>
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<td></td>
<td>4-h sample</td>
<td>24-h sample</td>
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<tr>
<td>1</td>
<td>177</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>147</td>
<td>17</td>
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<td>8</td>
<td>91</td>
<td>96</td>
</tr>
<tr>
<td>12</td>
<td>283</td>
<td>20</td>
</tr>
<tr>
<td>15</td>
<td>88</td>
<td>64</td>
</tr>
</tbody>
</table>

*Compounds (ZK-703 or fulvestrant at 10 mg/kg) were injected subcutaneously into mice (groups of six) daily. Serum samples were taken from three mice in each group 4 and 24 hours after the dose given on the indicated days, and serum concentrations of the drugs were analyzed by liquid chromatography/mass spectroscopy/mass spectroscopy (LC/MS/MS). Blood had to be pooled to allow analytical analysis; consequently, each value represents the mean for three mice.
The next question was whether the strong reduction in ER expression caused by treatment with pure antiestrogens would improve long-term disease stabilization. As Fig. 3, A, shows, MCF7 tumors in mice treated with fulvestrant grew more slowly than those in mice treated with tamoxifen. The growth control with ZK-703 was even stronger (P < .001); it inhibited tumor growth for more than 200 days (time to reach the threshold of 60 mm²), whereas tumors in mice treated with fulvestrant began progressing by 70 days.

We carried out a similar experiment using the ZR75-1 human breast cancer model. These tumors are strongly estrogen dependent but were only marginally inhibited by tamoxifen treatment (Fig. 3, B)—that is, they displayed partial tamoxifen resistance. Subcutaneous administration of ZK-703 at 10 mg/kg completely prevented tumor growth in the ZR75-1 model, inhibiting tumor progression for more than 180 days (P < .001).

Tumor Growth in ZK-703–Treated MCF7/TAM Xenograft Mice

We next characterized ZK-703 effects on the MCF7/TAM breast cancer model, which was created by passaging tumor material from MCF7-bearing mice that had relapsed under tamoxifen therapy. This breast cancer model is strongly estrogen dependent (ovariectomy inhibits tumor growth nearly completely; data not shown) but is resistant to tamoxifen (Hoffmann J, Schneider MR: unpublished results). As Fig. 4 shows, fulvestrant initially inhibited tumor growth (70 days) but did not confer long-term growth suppression; by contrast, ZK-703 was distinctly more potent (P < .001) with respect to time to progression (>180 days). In fact, the tamoxifen-resistant tumors appeared to be as sensitive as the tamoxifen-sensitive MCF7 tumors to ZK-703.

Characterization of ZK-253 in Human Breast Cancer Models

ZK-253 was designed in the course of structural optimization of ZK-703 (data not shown) in an effort to find a compound that would maintain strong antiproliferative activity against breast cancer, even when given orally. ZK-253 was tested orally and subcutaneously at doses of 1 and 10 mg/kg and showed maximal activity at 10 mg/kg when given either orally or subcutaneously (data not shown). ZK-253 administered orally at 10 mg/kg conferred long-term growth control of MCF7 tumors (Fig. 5). This compound also conferred long-term growth control of MCF7/TAM tumors when given subcutaneously and showed similar activity to ZK-703 in this model system (Fig. 6, A). We analyzed ER protein levels in MCF7/TAM tumor tissue from each treatment group and found, surprisingly, that tumors from groups that relapsed (i.e., whose tumor sizes were close to or above the threshold of 60 mm²) under therapy with tamoxifen,
raloxifene, or fulvestrant still expressed ER at levels comparable to the control. By contrast, treatment with both ZK-253 and ZK-703 reduced ER levels (Fig. 6, B).

We also evaluated the effect of ZK-253 in the ZR75-1, T47D, BT474, and MV3366 panel of human breast cancer xenografts. In the MV3366 model (Fig. 7) and in all the other models (data not shown), tumor growth inhibition by ZK-253 was long lasting and superior to that conferred by the other tested compounds. To demonstrate the improved oral activity of ZK-253 in comparison with ZK-703, both compounds were given orally. Indeed, ZK-703 at 50 mg/kg was less active than ZK-253 at 10 mg/kg.

**ZK-703 and ZK-253 Effects in Chemically Induced Rat Breast Cancer Models**

The activity of ZK-703 and ZK-253 was also studied in rats with NMU- and DMBA-induced breast tumors. In both models, ovariectomy resulted in strong inhibition of tumor growth (data not shown). Because ZK-703 has a higher oral bioavailability in rats than in mice, both antiestrogens were administered orally. In the DMBA model, oral administration of ZK-703 and ZK-253 (3 mg/kg daily for 4 weeks) inhibited tumor growth statistically significantly ($P < .001$) in comparison with vehicle-treated control rats. After 4 weeks of treatment, mean tumor weight in ZK-703–treated rats was 4.4 g (95% CI $= 1.1$ to $7.7$ g), and for ZK-253–treated rats, it was 3.1 g (95% CI $= 1.2$ to 5.0 g), whereas that in control rats was 24.9 g (95% CI $= 16.7$ to 33.1 g). Similarly, ZK-703 (given orally at 3 mg/kg daily for 4 weeks) produced a statistically significant ($P = .031$) 75% growth inhibition (mean tumor weight: 3.9 g, 95% CI = 1.2 to 6.6 g) relative to vehicle-treated control (mean tumor weight: 20.5 g, 95% CI = 9.7 to 31.3 g) in the NMU model, whereas fulvestrant (given orally at 3 mg/kg daily for 4 weeks) produced...
Antiestrogens of the SERM type, such as tamoxifen, represent an important therapeutic opportunity: They are active against breast cancer and have the favorable side effect of preventing osteoporosis (2). However, there are two principal limitations to the use of the currently available SERMs. The first is their limited specificity, which results from the mixed agonist and antagonist profile; the second is the early onset of resistance, or loss of activity, which also might be related to the limited specificity. A new generation of pure antiestrogens, the SERDs, was therefore developed to overcome these limitations. In this article, we have shown that two of these new antiestrogens, ZK-703 and ZK-253, strongly inhibited the growth of estrogen-responsive tumors and led to destabilization of the ER. Both compounds were more effective than tamoxifen and fulvestrant, and both prevented tumor progression in tamoxifen-resistant breast cancer models.

ZK-703 is a pure estrogen antagonist that causes efficient destabilization of the ER protein in T47D breast cancer cells (Parczyk K; unpublished results). The concentration required for destabilization (1.29 × 10⁻¹⁰ M) is lower than the concentration required for antiproliferative activity (IC₅₀ = 2.1 × 10⁻⁹ M; Table 1), suggesting a strong contribution of receptor destabilization onto tumor cell growth inhibition.

Pharmacokinetic investigations in mice revealed that ZK-703 can achieve serum levels similar to those of fulvestrant when both antiestrogens are given with the same subcutaneous schedule (Table 2). The 24-hour fulvestrant serum levels in mice injected daily subcutaneously with fulvestrant at 10 mg/kg ranged from 50 to 100 ng/mL (Table 2), up to five times higher than therapeutic serum levels of fulvestrant after an intramuscular injection of 250 mg in humans, as reported by Howell et al. (16). These data thus suggest that, in our experiments, therapeutic serum levels of ZK-703 and fulvestrant comparable to those seen in humans were achieved in mice, and they raise the possibility that effects similar to those seen on tumor growth in mice may be seen on tumor growth in humans.

The reasons for the improved efficacy of ZK-703 against tumor growth in comparison with that of tamoxifen and fulvestrant need to be explored in more detail. At present, only provisional explanations can be given. ZK-703 is more potent than tamoxifen and fulvestrant, and ER destabilization is more pronounced after treatment with ZK-703 than after treatment with either of these drugs (Fig. 2). We are currently carrying out experiments to determine whether higher tumor concentrations can be achieved with ZK-703 than with fulvestrant or whether differences in the response to transcription or signal transduction pathways might be responsible for the superior activity of ZK-703.

The promising pharmacologic profile of ZK-703 appears to provide an advantage in the treatment of breast cancer. By using the MCF7 breast cancer model in nude mice we found, as did Osborne et al. (17), that treatment with fulvestrant resulted in prolonged tumor growth control in comparison with tamoxifen, although we used slightly different experimental conditions (i.e., different tumor cell clones, dosing, and vehicles). Furthermore, we found a distinctly improved time to tumor relapse under ZK-703 therapy compared with that under fulvestrant therapy (30 weeks versus 10 weeks). We also found that ZK-703 treatment inhibited progression of tumors of the ZR75-1 human breast cancer model, which is strongly estrogen dependent but partly resistant to tamoxifen, for more than 6 months (Fig. 3, B).

From results of the first phase III studies with fulvestrant (18,19), it is evident that there is some cross-resistance between antiestrogens in patients (i.e., pure steroidal antiestrogens versus triphenylethylene-based antiestrogens, such as tamoxifen). To investigate cross-resistance to tamoxifen, we used an in vivo human breast cancer xenograft model, MCF7/TAM, that has acquired resistance to tamoxifen, created as described by Gottardis and Jordan (20). Treatment with ZK-703 resulted in prolonged growth control of these MCF7/TAM breast tumors, and resistance was not observed. From this experiment, we conclude that tumors that were resistant to tamoxifen were not cross-resistant to ZK-703; therefore, this drug should be active against tumors that have developed a resistance to the triphenylethylene-based antiestrogens. Fulvestrant was only moderately active in this model.

Although the activity of ZK-703 is encouraging, this compound was less effective when administered orally than when administered subcutaneously. By contrast, ZK-253, a structurally optimized form of ZK-703, retained the antiproliferative activity of ZK-703 against breast cancer, even when administered orally. Orally administered ZK-253 also had similar activity to subcutaneously given ZK-703 in the MCF7 model (Fig. 5).

Treatment with either new antiestrogen resulted in measurable ER destabilization in the MCF7/TAM breast cancer model (Fig. 6, B). By contrast, ER expression was higher in tumors of ovariectomized mice than in those of control mice and was not changed after 6 weeks of treatment with tamox-
ifen or fulvestrant. It is surprising that treatment groups that relapsed under therapy with either SERM or the pure antiestrogen fulvestrant (Fig. 6, B) still expressed the ER, because loss of the ER has been suggested to be a major factor in the development of resistance (21). Our data suggest that the lowered ER levels in MCF7 tumors caused by treatment with pure antiestrogens (as shown in Fig. 2, B) contribute to the long-term inhibition of estrogen-dependent tumor cells. If, however, the antiestrogen no longer inhibits ER expression, as we observed with fulvestrant in the MCF7/TAM tumors (Fig. 6, B), the result might be an acquired cross-resistance to tamoxifen. Although this hypothesis is obvious, we note that there is some statistical uncertainty; because of experimental limitations, we were able to measure ER levels only once under treatment and, in some cases, had to measure ER from pooled samples. Because expression of the PR is regulated by the ER, we determined PR expression to investigate the functional status of the ER. The ER was functional in the MCF7 model because the PR was induced by treatment with estrogen and the partial estrogen agonistic tamoxifen, whereas it was suppressed by treatment with pure antiestrogens and ovariectomy.

Broad direct comparison with other antiestrogens [i.e., tamoxifen, fulvestrant, and EM-800 (22)] in hormone-sensitive breast cancer models confirmed our pharmacologic characterization of ZK-253. Tumor growth inhibition by this new pure antiestrogen was long-lasting and superior to that conferred by the other compounds. We found that ZK-253 was distinctly superior to the other antiestrogens in all of the hormone-sensitive breast cancer models we screened (data not shown).

Experimental mammary tumors induced by the chemical carcinogens NMU and DMBA in female rats have similarities to breast cancers in women. The NMU-induced mammary tumor has some advantages over the DMBA model in that it is less dependent on prolactin for its growth than the DMBA tumor and is therefore more similar to human breast cancer (23). Both tumors are aggressive mammary carcinomas that are ER and PR positive and whose growth is strongly dependent on ovarian hormones (24). Known antiestrogens and aromatase inhibitors are effective in these tumor models (25) and, in this article, we have shown that both ZK-703 and ZK-253 are also potent inhibitors of the growth of both the NMU- and DMBA-induced rat mammary carcinomas. Even at the low dose of 3 mg/kg, when the compounds were given daily and orally, a statistically significant tumor growth inhibition was observed.

To summarize, our preclinical data indicate that treatment with ZK-703 or ZK-253 inhibits the growth of human breast cancer xenografts and reduces expression of the ER. ZK-253 is the most potent orally applicable antiestrogen developed to date. This compound has broad activity in both antiestrogen-sensitive and antiestrogen-resistant breast cancer models. Most important, ZK-253 prevented further tumor progression in tamoxifen-resistant breast cancer models. Because the preclinical pharmacologic profile promises substantial clinical progress for the endocrine treatment of hormone-sensitive breast cancer by this new generation of pure antiestrogens, safety and tolerability will be evaluated in the next steps.

References

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