Combination therapy for treating breast cancer using antiestrogen, ERA-923, and the mammalian target of rapamycin inhibitor, temsirolimus

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Abstract

The effect of combinations of a mammalian target of rapamycin (mTOR) inhibitor, temsirolimus, and an estrogen receptor-α (ERα) antagonist, ERA-923, on breast carcinoma in culture and in a xenograft model has been studied. Phase III trials are underway using temsirolimus for several cancers. ERA-923 was studied in a phase I trial for tamoxifen refractory metastatic breast cancer and was shown to have good safety profiles. Combination of noninhibitory doses of temsirolimus with suboptimal doses of ERA-923 synergistically inhibited the growth of MCF-7 cells. Synergy was found across a wide range of doses and could also be achieved by combining temsirolimus with other antiestrogens such as raloxifene and 4-hydroxy-tamoxifen. In vivo combination of temsirolimus and ERA-923 at certain doses and schedules completely inhibited tumor growth, while individual agents were only partially effective. Although the mechanism underlying the synergism remains to be understood, the results were associated with the ability of temsirolimus to block the transcriptional activity mediated by ERα as well as an increase in G1 arrest when it was combined with ERA-923. Results demonstrated for the first time that the combination of temsirolimus and a pure antiestrogen has excellent anticancer activity in preclinical models and, therefore, may have clinical use in treating hormone-dependent tumors.

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Introduction

Breast cancer is a leading cause of female cancer deaths in the world (American Cancer Society, 2006). Although there is a variety of chemotherapeutic treatments for breast cancer, the disease is frequently marginally responsive, especially in the later stages (Smith et al. 2000, Bergh et al. 2001, Kim et al. 2001). The growth of some human breast cancer cells is under hormonal control. Substantial evidence suggests that estrogen promotes the development of breast cancer and stimulates tumor growth through estrogen receptor-α (ERα), which is a member of a large family of ligand-inducible transcription factors (McKenna & O’Malley 2001, Reid et al. 2002, Germain et al. 2003). Upon binding to its receptor, the ligand initiates the dissociation of heat shock proteins from the receptor, receptor dimerization, phosphorylation, and binding to DNA-response elements of target genes. After binding to DNA, ERα differentially regulates transcription of target genes with or without other transcription factors and coactivators/corepressors. Estrogen action can be partially blocked by antagonists (antiestrogens) by competing out estrogen binding to estrogen receptor (ER) without activating genes that promote cell growth. The antiestrogen tamoxifen (Tam) has been used as first-line therapy in the treatment and
management of breast cancer (Jaiyesimi et al. 1995, Kaufmann 1997, Jordan 1999). Unfortunately, the effectiveness of Tam therapy is hampered by its agonist activity in other tissues such as the uterus and side effects such as hot flushes as well as the frequent occurrence of Tam-resistant carcinoma (Horwitz 1993, Ring & Dowsett 2004). The reasons for Tam resistance, in most cases, are not well understood. In addition to the pharmacology of Tam, cross-talk between the ER and signal transduction pathways has been considered as another potential mechanism (Horwitz 1993, Ring & Dowsett 2004, Johnston 2005).

Many studies have shown that growth factors such as epidermal growth factor (EGF) and insulin growth factor (IGF) are able to activate ER in a ligand-independent manner via multiple downstream pathways such as the mitogen-activated protein kinase (MAPK), the phosphatidylinositol 3-kinase (PI3K), and p38 kinase (Johnston 2005). In cells that are resistant to long-term estrogen deprivation, ERα, human epidermal growth factor receptor 2 (HER2) and MAPK activities are enhanced, resulting in a near ten-fold increase in ER-mediated gene transcription (Martin et al. 2003). Furthermore, increased levels of HER2, epidermal growth factor receptor (EGFR), and phospho-p38 in Tam-resistant tumors and cancer specimens were also reported (Gutierrez et al. 2005). Therefore, it appears that targeting signal transduction pathways and developing optimal combinations of antiestrogens with other therapeutic agents may effectively circumvent the resistance and achieve better efficacy while reducing the side effects of Tam. For example, multiple agents including gefitinib, MAPK inhibitor UO126, and ER downregulator fulvestrant, which degrades the ER, have been shown to suppress both the growth and ER-mediated gene transcription in cells adapted to long-term estrogen deprivation (Martin et al. 2003).

Recently, the evidence of cross-talk between the mammalian target of rapamycin (mTOR) pathway and ER is emerging. deGraffenried et al. (2004) demonstrated that rapamycin restored Tam resistance in MCF-7 breast cancer cells overexpressing AKT. The estrogen-dependent growth of wild type MCF-7, aromatase-expressing MCF-7/Aro, and T47D breast cancer cells was inhibited effectively by a mTOR inhibitor, RAD001 (Boulay et al. 2005). Moreover, synergistic inhibition was observed when RAD001 was combined with the aromatase inhibitor, letrozole (Boulay et al. 2005). Rapamycin and cyclosporine were recently reported to target both ERα and ERβ to proteasome-mediated proteolysis, resulting in decreased estradiol (E2)-induced ER transactivation (Gougelet et al. 2005). Interestingly, a farnesyl transferase inhibitor, lonafarnib, was shown to potentiate Tam-mediated apoptosis in MCF-7 breast cancer cells (Basso et al. 2005). The results also support a potential role of mTOR in Tam-resistant tumors since lonafarnib inhibits Rheb farnesylation, a positive regulator of mTOR. Overall, these data suggest that combination of mTOR pathway inhibitors with inhibitors of various signaling pathways is a viable approach to treat breast cancers. In fact, combinations of the mTOR inhibitors temsirolimus and RAD001 with letrozole are now in clinical trials (Johnson et al. 2002).

Temsirimus, an ester of sirolimus, is a potent mTOR inhibitor that is under development as an antitumor agent (Gibbons et al. 2000, Dancey 2002, Elit 2002, Punt et al. 2003). Temsirolimus showed significant activity in vitro against a variety of cancer cells including MCF-7 breast cancer cells (Yu et al. 2001). Although the mechanism underlying the inhibition is not entirely clear, the data suggest that, like sirolimus, temsirolimus inhibited translation machinery involved in the regulation of G1- to S-phase transition in the cell cycle (Fingar & Blenis 2004, Gingras et al. 2004). Previously, in a transient transfection assay, we found that temsirolimus was able to inhibit the transactivation of ER in MCF-7 cells (Zhang et al. 2003). The results prompted us to investigate a possible interaction between temsirolimus and another potent antiestrogen under development, ERA-923 (Greenberger et al. 2001).

In this study, we demonstrate the efficacy of combining temsirolimus and ERA-923. This combination synergistically inhibited the growth of breast cancer MCF-7 cells through enhanced G1/S arrest. Our preliminary experiments also showed dramatic antitumor activity against an MCF-7 xenograft model. Furthermore, at the molecular level, we demonstrated that the observed synergistic effect was likely due to the combination effect on several cellular targets rather than on ER directly. The combination of temsirolimus with antiestrogen may efficiently improve current therapy and prevent the emergence of tumor resistance.

Materials and methods

Materials

All cell culture reagents were purchased from Invitrogen except for charcoal-stripped fetal bovine serum, which was obtained from Hyclone (Logan, UT, USA). Steady-Glo Luciferase Assay System was purchased from Promega. Apoptotic DNA-Ladder kit
was purchased from Roche Applied Science. pFR-Luc was purchased from Stratagene (La Jolla, CA, USA).

**Plasmid construction**

Gal4-ERα(242) was made by fusing the ligand domain of ERα (from amino acids 242 to 530) with the Gal4 DNA-binding domain. Briefly, PCR was performed to generate cDNA corresponding to the ligand-binding domain of ERα with the BamH1 site at the 5’-end and the Mlu I site at the 3’-end. The PCR fragment was inserted into multiple cloning sites of the pM vector from Clontech to create Gal4-ERα(242).

**Cell culture and growth assay**

MCF-7 cells obtained from Dr Robert Clarke (Georgetown University Medical Center, Washington DC, USA) were maintained in IMEM medium supplemented with 10% fetal bovine serum. BG-1 human ovarian cancer cells were maintained in IMEM medium supplemented with 10% fetal bovine serum, 2 μg/ml insulin, and 1% nonessential amino acids. T47D and Cos-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum. All cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Before the experiment, cells were plated in 96-well plates at a cell density of 2500 cells/well in IMEM medium supplemented with 10% fetal bovine serum (Hyclone). The next day, cells were treated with various concentrations of anitestrogen and/or temsirolimus in regular media. Four days later, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (20 μl) (5 mg/ml) was added to each well and cells were incubated for an additional 4 h. The solution was then removed and 150 μl dimethyl sulfoxide added. The intensity of dark blue color was read at 540 nM with an automated plate reader.

**Transient transfection and luciferase assay**

MCF-7 cells were plated in 6-well plates at a density of 2×10⁵ cells per well in 2 ml phenol red-free IMEM medium containing 10% charcoal-stripped fetal bovine serum. The next morning, the medium was replaced with 2 ml IMEM. An indicated amount of DNA in 100 μl IMEM was mixed with 6 μl PLUS reagent (Invitrogen) and incubated at room temperature while 4 μl lipofectamine was mixed with 100 μl IMEM. After 30 min of incubation, the two mixtures were mixed together for 30 min and added dropwise to each well. After incubation with DNA for 4 h, 2 ml phenol red-free IMEM containing 10% charcoal-stripped fetal bovine serum was added. After 18 h, cells were harvested and plated in 96-well plates at a density of 10 000 per well and treated with the indicated chemicals for 6 h. Luciferase activity was determined using Promega’s Steady-Glo Luciferase Assay System. Briefly, after 6 h of treatment, 100 μl of Steady-Glo reagent was added to each well and luminescence determined in a luminometer (1450 MicroBeth Counter, PerkinElmer, Boston, MA, USA) after 5 min of gentle mixing.

**Western blot analysis**

Cells were harvested in M-PER reagent purchased from Pierce (Rockford, IL, USA). Thirty micrograms protein were electrophoresed on a 12% SDS-PAGE gel, transferred to polyvinylidene fluoride using a BioRad liquid transfer apparatus. The western analysis was performed with enhanced chemiluminescence purchased from Pierce using the F-10 antiERα antibody obtained from Santa Cruz (Santa Cruz, CA, USA) at 1 μg/ml and the horseradish peroxidase-conjugated antimouse secondary antibody (BD Biosciences, San Diego, CA, USA) diluted 1:4000. Phosphorylation of ERα was detected using antiphospho-ER antibodies purchased from Cell Signaling Technology (Danvers, MA, USA).

**Cell-cycle analysis**

For each assay, 5×10⁵ cells were plated in each well of a 6-well plate and treated with temsirolimus, ERA-923, or a combination of both. After 4 days, the medium was collected; cells were washed with PBS and trypsinized. The collected medium and the harvested cells were combined and centrifuged. The pellet was washed two times with PBS, fixed in 80% cold ethanol for at least 1 h at −20 °C, washed with PBS again, and centrifuged. The resultant pellet was incubated with 500 μl of propidium iodide (15 μg/ml) (Calbiochem, San Diego, CA, USA) and 20 mg/ml of RNase (Sigma) for at least 1 h at room temperature, and analyzed by flow cytometry using the Cell Quest program of the FACS Calibur machine (BD Biosciences).

**Synergy analysis**

The combinations were evaluated using a 3D shareware, MacSynergy II, developed by Prichard and Shipman (1990). Briefly, theoretical additive interactions were calculated from the dose–response curves of each individual drug. The calculated additive surface was then subtracted from the experimental surface to obtain a synergy surface representing percentage inhibition above the calculated additive value. Any peak above the 0% plane suggests synergy.
Likewise, any peak below the 0% plane is indicative of antagonism.

**In vivo studies**

Five- to six-week-old female ovariectomized athymic nu/nu mice (Charles River Labs, Wilmington, MA, USA) weighing 20.0–23.0 g were used. The animals were housed 5 to a cage in a Microisolator Open Rack System (Lab Products, Seaford, DE, USA). Each mouse received a 17β-estradiol pellet (0.72 mg/pellet-60-day release) from Innovative Research (Southfield, MI, USA). The pellets were injected with a 10-gauge trochar (Innovative Research) into the lateral side of the neck between the ear and the shoulder 1–2 weeks prior to tumor injection. Control groups were 15 mice/vehicle, whereas the drug groups were 10 mice/drug. Tumors were measured weekly by means of solar calipers (Cole-Parmer Instruments, Vernon Hills, IL, USA) and tumor weights were estimated from tumor diameters by the following formula:

\[
\text{Tumor weight (mg)} = \frac{\text{tumor length (mm)} \times \text{tumor width (mm)}^2}{2}
\]

Mice were euthanized 36 days after tumor injection by CO₂ inhalation. Ten million cells suspended in IMEM with 2% serum at a 1:1 ratio with Matrigel (Collaborative Research, Bedford, MA, USA) were injected subcutaneously into the mammary tissue of each mouse using a 1.0 ml tuberculin syringe with a 23 ¾-gauge needle. ERA-923 was dissolved in 1% Tween 80 (Sigma) and 0.9% NaCl injection USP (B. Braun Medical, Allentown, PA, USA). Drug was aliquoted into daily doses using 10.0 ml glass bottles and frozen at −20 °C until needed. Temsirolimus was made fresh using 5% ethanol, 4.9% Phosal (Donated by Maureen Harrison, Pearl River, NY, USA) and 0.1% Tween 80 in sterile water (Invitrogen). A measure of 0.2 ml of each drug was given orally starting the day after tumor implantation. ERA-923 was given daily for the duration of the experiment and temsirolimus was given every other day for the first 10 days. Vehicles used for the control group include vehicles from both drug-alone groups (ERA-923) and temsirolimus.

**In vitro whole cell ligand binding assay**

Lysates of MCF-7 cells were prepared by washing cells in isotonic-buffered saline followed by one wash with 1 mM Tris–HCl, 1 mM EDTA, pH 7.4. After cells were scraped on ice, they were homogenized using a Brinkman Polytron and lysates were centrifuged for 1 h at 100 000×g. The protein concentration of the resulting supernatant was determined by the Bradford method (BioRad). For binding assays in 96-well dishes, 100 μl supernatant (approximately 1–5 μg protein) were mixed with 10 μl 12 nM [³H]-17β-estradiol (70 Ci/mM; PerkinElmer) in the presence or absence of test compounds. After incubation for 2 h at room temperature, the unbound material was aspirated, the plate was washed three times with 300 μl PBS containing 1 mM EDTA, pH 7.4, and after aspiration, the liquid scintillation cocktail (PerkinElmer) was added to the wells. Radioactivity was determined using a liquid scintillation counter.

**ER–DNA-binding activity assay**

Nuclear extract was prepared using the Nuclear Extract kit (Active Motif, Carlsbad, CA, USA) and DNA binding was determined using TransAM ER kit (Active Motif). Assays were performed according to the manufacturer’s instructions.

**Results**

**Potent inhibition of MCF-7 cells by the combination of ERA-923 with temsirolimus**

The inhibitory activity of ERA-923, temsirolimus or a combination of both on the growth of MCF-7 cells was examined by MTT assay after the cells were treated with drugs for 4 days. Figure 1A shows that the percent inhibition increased with increasing concentrations of ERA-923. The IC₅₀ was estimated as 1 nM. Temsirolimus had no inhibitory effect at concentrations up to 10 nM (Fig. 1A). However, temsirolimus significantly potentiated the inhibitory activity of ERA-923. At all doses tested, combination treatment was more effective in inhibiting the growth than treatment with ERA-923 alone. For example, 0.25 nM ERA-923 inhibited only 10% of the growth. When combined with 10 nM temsirolimus (a noninhibitory dose), the percent inhibition increased nearly four-fold to 40%. Furthermore, treatment with 50 nM temsirolimus in combination with any of the doses of ERA-923 (0.25–5 nM) resulted in near complete inhibition of the growth of MCF-7 cells.

**Synergistic effect of ERA-923 with temsirolimus in inhibiting cell growth**

The potent inhibition by the combination of ERA-923 and temsirolimus suggested that the two drugs worked synergistically. A shareware program, MacSynergyII, was used to determine if the two drugs indeed synergistically inhibited the growth of cells. Two plates
of cells were treated identically with ERA-923, temsirolimus or in combination as shown (Fig. 1B). After 4 days, an MTT assay was performed and the data were analyzed by MacSynergyII. The 3D graph was plotted using the data with 95% confidence. Synergy was evident at any combination of the two drugs. However, synergy was more pronounced at low concentrations of ERA-923 (0.25–1 nM) in combination with high concentrations of temsirolimus (100–400 nM). The highest synergy observed was about 40% above the additive value (Fig. 1B).

Synergistic effect of temsirolimus with other antiestrogens in inhibiting cell growth

It was demonstrated that ERA-923 is a potent antiestrogen (Greenberger et al. 2001). We hypothesized that temsirolimus might also synergize with other antiestrogens in inhibiting cell growth. Growth of cells was inhibited in a synergistic manner by the combination of temsirolimus with either of the antiestrogens. The synergy volume (μM²%, total synergy observed under all peaks) that resulted from the treatment of temsirolimus in combination with ERA-923, raloxifene, or 4-OH-Tam was 138, 77 and 113, respectively. To eliminate potential errors introduced by the design of the experiment, we also combined temsirolimus with temsirolimus and ERA-923 as two control groups. The synergy volumes for each group were 17 and 10 respectively.

Potent inhibition of MCF-7 in xenograft model by ERA-923 plus temsirolimus

The effect of temsirolimus plus ERA-923 was tested in a xenograft model of human breast cancer to determine if temsirolimus can potentiate the effect of antiestrogen in vivo (Table 1). The inhibitory effect of temsirolimus was observed on day 14, while the inhibitory effect of ERA-923 was observed on day 28 as previously reported (Greenberger et al. 2001). The combination of both was superior to each agent given alone. For example, on day 36 ERA-923 or temsirolimus had a partial effect (approximately 35% inhibition of growth; growth inhibition = experimental value − 200 (baseline)/control value − 200). However, the combination of the drugs inhibited tumor growth at approximately 85%. Importantly, no signs of toxicity were observed with this drug combination.

Inhibition of ER transcriptional activity by ERA-923 and temsirolimus

The potential molecular mechanism for this synergistic effect was investigated. The effect of compounds on ER transcriptional activity was tested by using MCF-7 cells transfected with a reporter gene EREe1bLuc containing an estrogen-response element, a simple e1b promoter, and the luciferase gene 24 h before treatment. ERA-923 exhibited very potent antiestrogen activity with an IC₅₀ of 7 nM (Fig. 2A). Interestingly, we found temsirolimus
Table 1 Effect of ERA-923 and temsirolimus on MCF-7 in a mice xenograft model. Values are means ± s.e.m. (mg) of 10 mice

<table>
<thead>
<tr>
<th>Days after tumor implantation</th>
<th>Control treatment</th>
<th>5 mg/kg temsirolimus (PO; q2d days 1–9)</th>
<th>20 mg/kg ERA-923 (PO; qd days 1–35)</th>
<th>5 mg/kg temsirolimus plus 20 mg/kg ERA-923</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>495 ± 46</td>
<td>260 ± 18*</td>
<td>480 ± 91</td>
<td>290 ± 22*</td>
</tr>
<tr>
<td>21</td>
<td>789 ± 83</td>
<td>485 ± 53*</td>
<td>679 ± 127</td>
<td>331 ± 34*</td>
</tr>
<tr>
<td>28</td>
<td>1111 ± 134</td>
<td>704 ± 63</td>
<td>842 ± 163</td>
<td>398 ± 33*</td>
</tr>
<tr>
<td>36</td>
<td>1425 ± 179</td>
<td>1123 ± 134</td>
<td>993 ± 197*</td>
<td>498 ± 88*</td>
</tr>
</tbody>
</table>

Note. Control treatment is the combined effect of animals treated with vehicle for temsirolimus (Phosal), vehicle for ERA-923 (Tween), or vehicle for both drugs. The average tumor sizes of each control group did not significantly vary from each other and therefore were pooled. Statistical analysis was performed using log transformation of the data followed by ANOVA; pairwise comparisons were carried out. The single asterisk refers to statistical significance at P < 0.05 compared with the control group. The double asterisk (*) or */* symbol refers to statistical significance, or lack thereof, in a pairwise comparison with temsirolimus or ERA-923 given alone. The values in the temsirolimus plus ERA-923 group were statistically different compared with control at the P < 0.001 level.

also inhibited ER activity in this transcription assay (IC_{50} = 2 nM, Fig. 2A). Moreover, combination of temsirolimus and ERA-923 caused an additive effect (Fig. 2A). Since sirolimus was known to form a complex with the immunophilin FKBP12, which binds to mTOR and inhibits its activity, we asked if the inhibition of ER transactivation observed was mediated through the inhibition of the peptidyl–prolyl isomerase activity of FKBP12. The isomerase inhibitors FK506 and ascomycin were tested and neither inhibited ER transactivation (Fig. 2B and C).

**Temsirolimus did not affect ER directly**

The inhibition of ER transactivation prompted us to ask if temsirolimus affected ER directly through ER ligand and DNA binding. It was found that temsirolimus did not affect estrogen binding to ER in a whole cell-binding assay (Fig. 3A). Western analysis revealed that only ERA-923 inhibited 50% of the ERα protein level (Fig. 3B). It appeared that ERA-923 reduced the phosphorylation of Ser118 slightly after the ERα phosphorylation signal was normalized to its protein level (data not shown). No additional effect on ERα protein or phosphorylation level was detected when ERA-923 was combined with temsirolimus (Fig. 3B). Moreover, it was found that ER–DNA-binding activity was inhibited significantly by ERA-923, but not by temsirolimus (Fig. 3C). No additional inhibition was observed when both compounds were used (Fig. 3C).

**The effect of ERA-923 and temsirolimus on cellular signaling and cell cycle in MCF-7 cells**

The effect of ERA-923 and temsirolimus on several cellular targets was examined to understand the mechanisms underlying the synergy. Our experiments showed that treatment with temsirolimus reduced the phosphorylation of p70S6 kinase (p70S6K) as well as the expression of retinoblastoma protein (Rb) and cyclin D1 (Fig. 4A). ERA-923 alone modestly reduced the phosphorylation of Rb and the expression of cyclin D1 (Fig. 4A). E2F transcriptional factor 1 (E2F1) was not affected by either agent or a combination of both. Interestingly, inhibition of the phosphorylation of Rb by ERA-923 was more pronounced in the presence of temsirolimus (Fig. 4A). Surprisingly, ERA-923 at 10 nM alone also significantly inhibited the phosphorylation of p70S6K and the inhibition was greatly potentiated by temsirolimus (Fig. 4B).

The analyses of these cellular targets suggested that the growth phase was the primary target of ERA-923, temsirolimus or both in combination. Therefore, cell-cycle experiments were performed to confirm this notion. Both ERA-923 and temsirolimus inhibited cells from entering into S-phase in a dose-dependent manner (Fig. 5A). Moreover, a combination of ERA-923 and temsirolimus appeared to be more effective in preventing the progression to S-phase than either drug alone (Fig. 5B).

**Discussion**

In this study we show that the combination of antiestrogens with an mTOR inhibitor temsirolimus synergistically inhibited the growth of breast cancer MCF-7 cells *in vitro*. In an *in vivo* breast cancer model, the combination of both inhibitors was far more effective than each inhibitor alone. Moreover, we demonstrate for the first time that temsirolimus directly inhibited the transcriptional activity of ER via the mTOR pathway. Our findings indicate that it may be possible to reduce the toxicity and side effects of current antiestrogen treatment by combining lesser doses of antiestrogens such as Tam with temsirolimus.
To make sure that the combination effect is not limited to one cell type only, we tested two other ER-positive cell lines, breast cancer cells T47D and ovarian cancer cells BG-1. Synergistic inhibition was observed in both cell types when temsirolimus was combined with ERA-923, 4-OH-Tam, or raloxifene (Table 2). The only exception is that temsirolimus failed to potentiate the effect of 4-OH-Tam in BG-1 cells. This is perhaps not surprising since the agonistic effect of 4-OH-Tam in ovary is well documented (Lee et al. 2000, Mourits et al. 2001).

Combination of chemotherapeutic drugs is a common practice in cancer therapy. Although both Figure 2 Inhibition of ER transcriptional activity by ERA-923 and temsirolimus. MCF-7 cells transfected with an e1bERE luciferase reporter were treated with 10 nM estradiol and individual drugs as indicated. (A) Temsirolimus, ERA-923, and a combination of both. (B) FK506. (C) Ascomycin. Luciferase activity was determined after 6 h. Each value is the mean±s.d. of six samples.

Figure 3 Temsirolimus did not affect ER directly. (A) Effect of temsirolimus on the binding of estradiol to ERα. MCF-7 cells were incubated with 2 nM [3H]estradiol in the presence of increasing amounts of cold estradiol or temsirolimus. After three washes, cells were lysed and bound [3H]estradiol measured with a scintillation counter. Each value is the mean±s.d. of three samples. (B) Effect of ERA-923, temsirolimus, and a combination of both on ERα phosphorylation status and protein level. MCF-7 cells were cultured in medium containing 10% charcoal-stripped FBS and treated with estradiol (10 nM), temsirolimus (250 nM) and ERA-923 (10 nM). Cells were harvested after 24 h and ERα protein level and phosphorylation (Ser118) were examined by western analysis. Actin was also probed as a control. (C) Effect of ERA-923 and temsirolimus on ER–DNA binding. Nuclear extracts were prepared from MCF-7 cells treated with estradiol with and without temsirolimus. DNA binding activity was determined using a TransAM kit from Active Motif. Data are means±s.e.m. of three experiments carried out in triplicate. *P<0.05 versus estradiol (E2)-treated group; **P<0.05 vs the group treated with E2 and temsirolimus.
ERA-923 and temsirolimus appeared to be cytostatic in MCF-7 cells in our study and were reported to arrest cells at G1-phase (Owa et al. 2001, Carraway & Hidalgo 2004, Fingar & Blenis 2004), it is critical to know whether the combination of drugs would cause apoptosis. Thus far, we have found no evidence of apoptosis (data not shown) with several detection methods including FACS analysis, caspase assay (ApoOne kit, Promega), Cell Death Detection ELISA (Roche), and Annexin Apoptosis Detection kit (Sigma), suggesting that the combination may only affect the proliferation of cells, which may reflect a slower rate of growth. FACS analysis confirmed that both ERA-923 and temsirolimus inhibited cells from entering into S-phase in a dose-dependent manner (Fig. 5A). Additionally, the combination of ERA-923 and temsirolimus prevented the progression to S-phase more effectively than either drug alone (Fig. 5B). It is

Figure 4 Effect of temsirolimus and ERA-923 on cellular signaling. MCF-7 cells were treated for 16 h with temsirolimus (50 nM), ERA (10 nM), or both as marked. Cells were harvested and western analysis performed on multiple cellular targets with their correspondent antibodies as marked. (A) Effect of temsirolimus and ERA-923 on multiple cellular targets. (B) Effect of temsirolimus and ERA-923 on phospho-p70S6K.

Figure 5 Effect of temsirolimus and ERA-923 on S-phase. MCF-7 cells were cultured in IMEM containing 10% charcoal-stripped FBS. After 24 h, cells were treated with various reagents as indicated. Cells were subjected to FACS analysis after 8 h of treatment. (A) Effect of individual compound on S-phase. *P<0.05 vs temsirolimus-treated group; **P<0.05 vs ERA-923-treated group. (B) Effect of combination treatment on S-phase. *P<0.05 vs E2-treated group; **P<0.05 vs corresponding temsirolimus-treated group; ***P<0.05 vs ERA-923-treated group.
Table 2 Average volume of synergy produced by antiestrogens and temsirolimus

<table>
<thead>
<tr>
<th>Combination</th>
<th>Synergy volume (μM²%)</th>
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</thead>
<tbody>
<tr>
<td>Temsirolimus + ERA-923</td>
<td>55.4</td>
</tr>
<tr>
<td>Temsirolimus + raloxifene</td>
<td>59.4</td>
</tr>
<tr>
<td>Temsirolimus + 4-OH-Tam</td>
<td>55.8</td>
</tr>
</tbody>
</table>

Data from Fig. 1 were analyzed by a 3D shareware, MacSynergy II developed by Prichard and Shipman (1990). Concentration of each drug used: temsirolimus (0–400 nM); ERA-923 (0–5 nM); raloxifene (0–5 nM); 4-OH-Tam (0–100 nM). ER negative Cos-1 cells were used as an additional control. Synergy volume for the combination of temsirolimus and ERA-923 in Cos-1 cells was 6.4. Synergy volume for the combination of compounds themselves (ERA-923 + ERA-923; temsirolimus + temsirolimus) was less than 5.5.

worth noting that two recent studies reported weak apoptosis associated with rapamycin treatment, which is not evident in our study. The difference may be due to either the pharmacological difference between rapamycin and temsirolimus or different concentrations used in the studies (Basso et al. 2005, Gougelet et al. 2005).

To exclude the possibility that temsirolimus affects ER activity through its effect on FK506 binding protein (FKBP) isomerase activity, we tested FK506 and ascomycin, both of which inhibit FKBP12 isomerase activity but not mTOR activity (Ivery & Weiler 1997, Mollison et al. 1998, Choi et al. 2002, Huang et al. 2003). Both FK506 and ascomycin failed to inhibit ER transcripntional activity, suggesting that inhibition of mTOR kinase activity and subsequent inhibition of the mTOR pathway is indeed responsible for the loss of ER transcriptional activity.

mTOR controls multiple cellular signalings (Rohde et al. 2001, Vogt 2001, Dennis & Thomas 2002, Asnaghi et al. 2004, Dutcher 2004). Our study demonstrated that ERA-923 inhibited the phosphorylation of Rb and p70S6K and the expression of Cyclin D1 while temsirolimus reduced the expression of Rb. However, a combination of temsirolimus and ERA-923 showed more than an additive effect on p70S6K and Rb phosphorylation, suggesting that these targets participated in the inhibition of ER transcriptional activity.

The mechanisms by which temsirolimus potentiated the effect of antiestrogens remain to be elucidated. During our preparation of the manuscript, deGraffenried et al. (2004) and Gougelet et al. (2005) reported that transactivation of ER is inhibited by temsirolimus and rapamycin respectively, which is consistent with our results. Our study utilizing the endogenous ER of MCF-7 cells as well as transiently transfected Gal4-ERα(242), which comprises the ER dimerization domain and ligand-binding domain, is not only in agreement with their observation, but also identified that the dimerization domain and ligand-binding domains are sufficient to confer a temsirolimus effect (data not shown). However, in our study, temsirolimus failed to inhibit the phosphorylation of ERα on Ser118 (Fig. 3B) as reported by deGraffenried et al. (2004). The difference is likely due to their use of a cell line expressing constitutive AKT. Since we did not observe any effect of temsirolimus on estrogen binding (Fig. 3A), ERα protein level (Fig. 3B), phosphorylation status (Fig. 3B), or ER–DNA binding (Fig. 3C), future studies on the other possible mechanisms such as involvement of coregulators of ERα are warranted.

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