Inhibition of mTOR Activity Restores Tamoxifen Response in Breast Cancer Cells with Aberrant Akt Activity

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ABSTRACT

The Akt kinase is a serine/threonine protein kinase that has been implicated in mediating a variety of biological responses. Studies show that high Akt activity in breast carcinoma is associated with a poor pathophenotype, as well as hormone and chemotherapy resistance. Additionally, high Akt activity is associated with other features of poor prognosis. Thus, a chemotherapeutic agent directed specifically toward tumors with high Akt activity could prove extremely potent in treating those breast tumors with the most aggressive phenotypes. Several studies have demonstrated that rapamycin, which inhibits mammalian target of rapamycin (mTOR), a downstream target of Akt, sensitizes certain resistant cancer cells to chemotherapeutic agents. This study evaluated the efficacy of mTOR inhibition in the treatment of tamoxifen-resistant breast carcinoma characterized by high Akt activity. We found that MCF-7 breast cancer cell lines expressing a constitutively active Akt are able to proliferate under reduced estrogen conditions and are resistant to the growth inhibitory effects of tamoxifen, both *in vitro* as well as *in vivo* in xenograft models. Cotreatment with the mTOR inhibitor rapamycin *in vitro*, or the ester of rapamycin, CCI-779 (Wyeth) *in vivo*, inhibited mTOR activity and restored sensitivity to tamoxifen, suggesting that Akt-induced tamoxifen resistance is mediated in part by signaling through the mTOR pathway. Although the mechanism underlying the synergism remains to be understood, the results were associated with rapamycin's ability to block transcriptional activity mediated by estrogen receptor α, as assessed by reporter gene assays with estrogen-responsive element luciferase. These data corroborate prior findings indicating that Akt activation induces resistance to tamoxifen in breast cancer cells. Importantly, these data indicate a novel mechanism for tamoxifen resistance and suggest that blockage of the phosphatidylinositol 3′-kinase/Akt signaling pathway by mTOR inhibition effectively restores the susceptibility of these cells to tamoxifen. These data may have implication for future clinical studies of mTOR inhibition in breast carcinoma.

INTRODUCTION

Tamoxifen, which functions as a cell type-specific antiestrogen, has dominated endocrine treatment of breast cancer for over two decades with demonstrated efficacy in metastatic breast cancer, adjuvant therapy, preoperative treatment, ductal carcinoma *in situ* and chemoprevention (1). However, 50% of all estrogen receptor α (ER-α)-positive breast cancer patients present with *de novo* tamoxifen resistance, and almost all initial responders eventually develop resistance (2). The mechanisms by which this resistance occurs remain unclear. It has been previously shown that growth factors such as epidermal growth factor, insulin-like growth factor, and heregulin confer estrogen-independent growth properties to ER-α–positive breast cancer cells (3). Several studies (4) have now demonstrated that these estrogen-independent growth properties are mediated in part through the serine/threonine protein kinase B or Akt (PKB/Akt). In addition, it has been shown that PKB/Akt can protect breast cancer cells from tamoxifen-induced apoptosis (4, 5). These data suggest a significant role for the PKB/Akt signaling pathway in hormone-refractory breast cancer.

PKB/Akt is activated in cells exposed to diverse stimuli such as hormones, growth factors, and extracellular matrix components (6, 7). The PKB family of serine/threonine protein kinases have been implicated in mediating a variety of biological responses that are central to the process of oncogenic transformation of mammalian cells, including inhibiting apoptosis and stimulating cellular growth (reviewed in ref. 8). Aberrant activation of the PKB/Akt pathway can suppress the apoptotic response, undermine cell cycle control, and selectively enhance the production of key growth and survival factors (9). Multiple laboratories have now demonstrated that the phosphatidylinositol 3′-kinase (PI3k)/Akt pathway provides these cell signals, in part, through activation of the mammalian target of rapamycin (mTOR; refs. 10, 11).

mTOR is a serine-threonine kinase that is activated by Akt and regulates the function of the transcriptional regulators $p70^{s6}$ kinase and 4E-BP1 (reviewed in ref. 12). In preclinical studies, rapamycin resulted in G_1 cell cycle arrest, induced apoptosis, and had antiproliferative effects in several cancer models. An ester of rapamycin, CCI-779, is currently being developed for the treatment of patients with cancer, including breast cancer.

Received 1/7/04; revised 7/6/04; accepted 8/20/04.
Grant support: Susan G. Komen Foundation Grant PDF 2000 655 (L. deGraffenried).
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We have recently demonstrated (13) that rapamycin is capable of restoring the susceptibility of prostate cancer cell lines with high Akt activity to chemotherapy. In this current study, we evaluated the ability of rapamycin and CCI-779 to restore tamoxifen sensitivity to refractory breast cancer cells with high Akt activity. We found that *in vitro*, breast cancer cells refractory to tamoxifen as a single agent, when cotreated with low levels of rapamycin, demonstrated a dose-dependent growth inhibition response to tamoxifen similar to that observed in tamoxifen-sensitive cells treated with tamoxifen alone. Similarly, in mouse xenograft experiments, breast cancer cell tumors refractory to tamoxifen due to high levels of Akt activity demonstrated a significant level of growth inhibition when the mice were administered both tamoxifen and CCI-779 together.

MATERIALS AND METHODS

Cell Lines. The parental MCF-7 breast cancer cells were obtained from the American Type Culture Collection and maintained in Improved Minimal Essential Medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO) and 6 ng/mL bovine insulin (Sigma). Stable transfectant cell lines were maintained in the same Improved Minimal Essential Medium treated with 400 mg/L G418.

Generation, Selection, and Analysis of Stable Transfectants. The expression plasmid for the myristoylated, constitutively active Akt1 (myrAkt Δ4-129) has been described previously (14). This plasmid produces an Akt1 protein M_r ~46,000 compared with the endogenous Akt1 of M_r 64,000 because the PH domain is replaced with the *v-src* myristoylation sequence. The MCF-7 cell line was transfected with either the Akt plasmid or with an empty pCDNA3.1(+) vector (Invitrogen, Carlsbad, CA) as a control, with FuGene 6 (Boehringer Mannheim, Indianapolis, IN). One day after transfection, cells were placed into the selection medium containing 1.0 mg/mL G418 (Life Technologies, Inc.). Twenty-one days after selection, individual G418-resistant colonies were subcloned. Protein expression was analyzed by Western blot analysis with antibodies for both phosphorylated and total Akt (Cell Signaling Technology, Beverly, MA) and the T7 epitope (Novagen, Madison, WI).

Western Blot Analysis. For clonal selection, after a 24-hour incubation in growth media, cells were washed and treated with Earle's salts containing 2 mmol/L L-glutamine as unstimulated control or 100 nmol/L insulin for 24 hours. Cells were harvested in 1× lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 120 mmol/L NaCl, 1% NP40, 1 mmol/L EDTA (pH 8.0), 5 mmol/L EGTA (pH 7.5), 50 mmol/L NaF, 40 mmol/L β-glycerolphosphate, 100 μmol/L Na orthovanadate, 1 mmol/L benzamidine, and protease inhibitor mixture]. Protein lysates were subjected to immunodetection with antibodies to first phosphorylated and then total Akt (Cell Signaling Technology), ER-α (6F11 monoclonal antibody, Novocastra Ltd., Newcastle upon Tyne, United Kingdom), and finally actin (Santa Cruz Biotechnology, Santa Cruz, CA) for a loading control. Evaluation of p70s6k and ER-α status was performed on lysates from cells treated 24 hours with serum-free media, media with 10% FBS, 10% FBS media with 10^{-7} mol/L 4-OH tamoxifen (Calbiochem, San Diego, CA), 20 nmol/L rapamycin (Sigma), a combination of tamoxifen and rapamycin, or 100 nmol/L insulin, with antibodies to phospho-p70s6 (Cell Signaling Technology) and phosphorylated ER-α 118 (Cell Signaling Technology). Tumor lysates were obtained using the 1× lysis buffer described above. Antibodies for total and phosphorylated 4-EBP1 were obtained from Cell Signaling Technology. Signal detection was done with the enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

Kinase Assays. Kinase activity assays were used to measure the effects of treatment on Akt activity, as has been described previously (15). Briefly, after 24-hour eicosapentaenoic acid and/or insulin treatment, cells were lysed in 1× lysis buffer and total Akt immunoprecipitated with 1 μg of total Akt antibody (Santa Cruz Biotechnology). Immunocomplexes were incubated with 5 μg of Akt substrate, a RPRAATF sequence peptide (α Diagnostic International, San Antonio, TX) and ^{32}ATP (PerkinElmer, Boston, MA). ATP incorporation was measured by scintillation counting.

Growth Proliferation Assay. Cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) dye conversion at 570 nm following manufacture's instructions. Briefly, cells were seeded 5×10^3 per well in a 96-well flat-bottomed plate. Cells were allowed to grow 24 hours, then placed in serum-starved conditions for 18 hours. Cells were then treated with increasing concentrations of tamoxifen alone or in combination with 20 nmol/L rapamycin (Sigma), as indicated, in the presence of 10% FBS supplemented with 6 ng/mL insulin. After 96 hours of continuous treatment, 20 μL of MTT (5 mg/mL in PBS) were added to each well. After 3 hours of incubation at 37°C, cells were lysed by the addition of 0.1 N HCl in isopropanol.

Luciferase Reporter Assays. Estrogen receptor transcriptional activity was assessed using a luciferase reporter gene driven by a 3× ERE-tk promoter (a kind gift from Dr. Arun Roy, UT Health Science Center at San Antonio, San Antonio, TX). Transient transfections were performed three times in triplicate wells. Cells were seeded in 6-well cluster plates (Falcon, Franklin Lakes, NJ) at a density of 2×10^5 cells per well 24 hours before treatment. Cells were then serum starved for 24 hours, followed by treatment with fresh serum-free media, Improved Minimal Essential Medium containing 10% FBS, 10% FBS media with 10^{-7} mol/L 4-OH tamoxifen, 10% FBS media with 20 nmol/L rapamycin, or 10% FBS media with both tamoxifen and rapamycin. Twenty-four hours after treatment, 3.0 μL of FuGene 6 transfection reagent (Roche, Indianapolis, IN) were used to transfect 1.0 μg of the pGL3-Basic 3× ERE-tk reporter construct. The plasmid pNull-Renilla (Promega, Madison, WI), an expression plasmid for the Renilla luciferase gene void of eukaryotic promoter or enhancer sequences, was cotransfected for transfection normalization. All transfections are reported as activity after normalization for Renilla expression. Forty-eight hours after transfection, luciferase activity was measured using the Dual Luciferase kit from Promega as per manufacturer's instructions. Shown is a combination of three independent experiments.

***In vivo* Studies.** All animal studies were conducted according to guidelines of the American Association of Laboratory Animal Care. One week before xenograft injection, 4 to 6-week-old athymic female mice were primed with 17β-estradiol (Innovative Research of America) applied s.c. in a biodegradable sustained-

release carrier binder (1.7 mg of estradiol per pellet) to promote tumor cell growth. Mice were inoculated with 1×10^6 myrAkt1 MCF-7 (clone 13) or control MCF-7 cells at each flank in PBS. Treatment was initiated when tumors reached ~200 mm^3. Tumor size was measured every other day with a caliper, and tumor volume was calculated by following formula: [length × $(width)^2$]/2. CCI-779 was initially dissolved in etomidate at a concentration of 50 mg/mL and then diluted to 2 mg/mL in 0.15 mol/L NaCl, 5% Tween 20, and 5% polyethylenglycol 400. Mice were treated with 10 mg/kg with fresh drug solutions every day five times the first week only. Tamoxifen was administered five times per week at 500 μg per animal. Control mice were injected with vehicle only. Treatment continued for 17 days. Growth inhibition was calculated by tumor volume of treated mice divided by volume of control mice (T/C).

Flow Cytometry. Cell cycle analysis of the cytostatic effect and sub-G_1 peak detection of the apoptotic effect was determined by flow cytometric analysis of 10^4 cells with the LysisII program on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Briefly, 24 hours after plating, cells were exposed to vehicle, tamoxifen (10^{-7} mol/L), rapamycin (20 nmol/L), or a combination of tamoxifen with rapamycin for 24 hours in 10% FBS media. Cells were collected after treatment, washed in PBS, and after cell centrifugation, cell pellets were stained with a propidium iodide (Sigma) staining solution containing 10 μg/mL propidium iodide, 1 μg/mL RNase, and 0.1% (v/v) Triton X-100. The cell suspension was incubated in the dark at 4°C for 18 hours before analysis. Determination of DNA content was based on a minimum total of 10,000 acquired events. Each experiment was done three times.

Apoptosis Assessment. Terminal deoxynucleotidyl transferase-mediated nick end labeling analysis was used to assess apoptosis in paraffin-embedded sections of the control and myrAkt1 xenograft tumors that were washed and then fixed in 10% formaldehyde-PBS. The assay was performed with TdT-FragEL DNA Fragmentation Assay kit (Oncogene, Cambridge, MA) per manufacturer's instructions. The percentage of apoptotic cells as determined by microscopic examination of terminal deoxynucleotidyl transferase-mediated nick end labeling-treated slides was expressed as an average of the ratio of apoptotic endothelial cells to the total number of endothelial cells in four random 0.011-mm^2 fields at ×400 magnification.

Statistics. For luciferase and MTT analysis, a Student *t* test was used. For tumor volume, a nonparametric equivalent (Kruskal-Wallis) test was used.

RESULTS

MCF-7 myrAkt1 Transfectants Show Constitutive Akt Activity. To better understand how specific components of the PI3k/Akt signaling pathway effect development of hormone-independent breast cancer, we developed MCF-7 breast cancer cell lines that express a myristoylated, constitutively active Akt1 kinase (myrAkt1 MCF-7), as well as MCF-7 cells with the empty vector alone (control MCF-7). As seen in Fig. 1*A*, MCF-7 control cells stably transfected with the pCDNA 3.1 vector displayed no phosphorylated endogenous Akt (M_r 64,000) under nonstimulated conditions (Earle's) but did demonstrate phosphorylation of the Ser^{473} residue when treated with 100 nmol/L insulin. Interestingly, this was in contrast to the MCF-7 cells stably transfected with the myrAkt1. As expected, the Ser^{473} of the myrAkt1 (M_r 46,000) was constitutively phosphorylated, but detectable levels of phosphorylated endogenous Akt were also observed even under mitogen-free conditions. No changes were observed in the expression levels of the ER-α, either between the control and Akt MCF-7 cells or upon treatment with insulin. The expression levels of the phosphorylated Akt correlated with the Akt kinase activity within the cell lines (Fig. 1*B*). Two myrAkt1 MCF-7 clones, 13 and 29, displayed a 17- and 7.5-fold, respectively, greater level of Akt kinase activity compared with those observed in the control cells under unstimulated conditions. Upon treatment with 100 nmol/L insulin, Akt kinase activity levels increased 5-fold in the control MCF-7 cells and ~2-fold in both the myrAkt1 MCF-7 cells. These results confirmed that the expression of the constitutively phosphorylated Akt resulted in mitogen-independent activity of Akt.

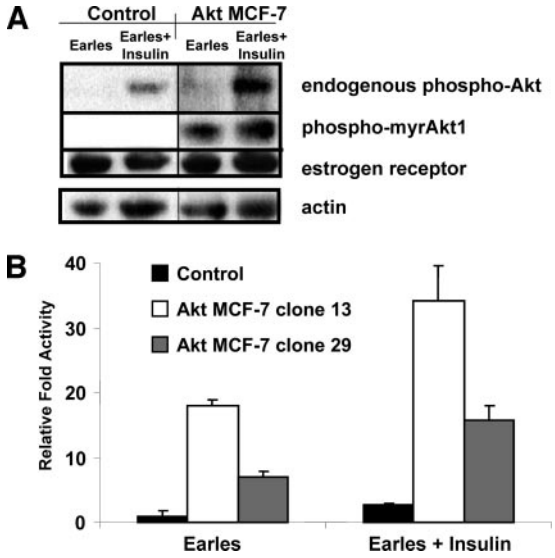


Fig. 1 Mitogen-independent activation of Akt. *A*, protein lysates from control and myristoylated Akt-transfected (Akt) MCF-7 cells before (Earle's) and after (+ insulin) stimulation with 100 nmol/L insulin were subjected to immunodetection with antibodies to the phospho Ser^{473} residue of Akt on both the endogenous (M_r 64,000, endogenous phospho-Akt), as well as the myrAkt1 transgene product (M_r 46,000, phospho-myrAkt1), the ER, and actin. *B*, Akt kinase activity of the control and myrAkt1 MCF-7 cells (clones 13 and 29) under nonstimulated (Earle's) and insulin-stimulated (Earle's + insulin) conditions. Kinase activity is presented as relative-fold activity compared with that in the unstimulated control cells and is a combination of three independent experiments.

Inhibition of mTOR Restores Tamoxifen Response *In vitro*. Several studies have now demonstrated that high levels of Akt activity confer resistance to tamoxifen (4, 5). To determine whether inhibition of downstream targets of Akt could restore tamoxifen response, we treated control MCF-7 cells (black), myrAkt1 MCF-7 cells expressing high levels of phosphorylated Akt (clone 13, white) and myrAkt1 MCF-7 cells expressing lower levels of phosphorylated Akt (clone 29, gray) with increasing concentrations of 4-OH tamoxifen in the absence (left panel) or presence (right panel) of 20 nmol/L rapamycin, an mTOR inhibitor

(Fig. 2). These MTT cell proliferation assays were conducted in 10% FBS supplemented with 6 ng/mL insulin.

Not surprisingly, the myrAkt1 MCF-7 cells with low expression levels of the transgene (clone 29) and the control MCF-7 cells both displayed dose-dependent sensitivity to tamoxifen at concentrations as low as 10^{-8} mol/L. Conversely, we found that the myrAkt1 MCF-7 cells with high Akt activity (clone 13) demonstrated a significant decrease in the growth inhibitory response to tamoxifen compared with that observed in the control or low Akt activity cells, in agreement with previously published reports (4, 5). Even at concentrations of 10^{-6} mol/L tamoxifen, the high myrAkt1 MCF-7 cells never demonstrated growth inhibition >10% compared with results with untreated cells. Similar results are obtained with CAMA-1 breast cancer cells that display high levels of Akt phosphorylation due to low expression levels of the Akt inhibitor, PTEN, and high expression levels of HER-2/neu (data not shown). Modulation of Akt activity in these cells by exposure to increasing concentrations of Akt inhibitors also modulates their response to tamoxifen.

Significantly, sensitivity to tamoxifen was restored when the cells were cotreated with 20 nmol/L of the mTOR inhibitor, rapamycin. By itself, the 20 nmol/L concentration of rapamycin did not significantly inhibit growth of either the control or the clone 13 MCF-7 cells. Surprisingly, the myrAkt1 MCF-7 cells with low Akt expression levels (clone 29) demonstrated significant response to treatment with 20 nmol/L rapamycin as a single agent, with growth inhibited to 65% that observed in nontreated cells. Cotreatment with rapamycin restored tamoxifen response in these cells to levels observed in the control cells, suggesting that mTOR signaling is an important mediator of Akt-induced tamoxifen resistance.

Rapamycin Treatment Inhibits ER-α–transcriptional Activity. To begin to determine the mechanism(s) by which rapamycin treatment restores sensitivity to tamoxifen *in vitro*, we investigated the phosphorylation state of ER-α in our control and myrAkt1 MCF-7 cells (Fig. 3*A*) and evaluated the ability of rapamycin to alter this state and inhibit ER-α–transcriptional activity, as assessed by reporter gene assays with an ERE-driven luciferase reporter (Fig. 3*B*). Several studies (refs. 4, 16; reviewed in ref. 17) have now shown that the AF1 domain of the ER is phosphorylated in response to mitogen treatment. Because Akt has been shown to phosphorylate and activate ER-α (4, 18), we investigated the phosphorylation state and the transcriptional activity of the ER-α in our myrAkt1 MCF-7 and control cells under unstimulated (serum free), as well as stimulated (10% FBS) conditions, and 10% FBS with 10^{-7} mol/L tamoxifen, 20 nmol/L rapamycin, and a combination of tamoxifen and rapamycin. Studies done evaluating the phosphorylation state of Ser^{167}, shown to be a primary target of Akt phosphorylation (4), were inconclusive. Because others have shown that Ser^{118} also appears to play a role in the activation of ER-α by Akt (18), we assessed the phosphorylation status of this residue of the ER-α. As seen in Fig. 3*A*, the myrAkt1 MCF-7 cells (A) demonstrated much higher levels of phosphorylated p70s6k (a downstream target of Akt) and phosphorylated ER-α 118 under unstimulated (serum-starved) conditions compared with those observed in the control cells (C). Ten percent FBS increased the observed levels of phosphorylation of p70s6k and ER-α in the control cells but had little effect in the myrAkt1 cells. As has been noted in previous studies (16), treatment of cells in 10% FBS with tamoxifen resulted in an increase in phosphorylation of the ER-α, as well as p70s6k in the control cells, but has little effect on the myrAkt1 cells. Not surprisingly, treatment with rapamycin abolished phosphorylation of p70s6k in both the control and myrAkt1 cells. Interestingly, rapamycin also appeared to decrease phosphorylation levels of ER-α 118 in both cell lines. Significantly, the combination of rapamycin with tamoxifen did not diminish phosphorylation levels of ER-α 118 any more than either tamoxifen or rapamycin as single agents. Insulin was used as a positive control and increased phosphorylation levels of both p70s6k and ER-α 118 in the control cells.

In Fig. 3*B*, we assessed ER-α activity by transiently transfecting both our control, as well as our myrAkt1 MCF-7 cells with an ERE-tk promoter luciferase reporter construct and measuring luciferase activity under nonstimulated (serum

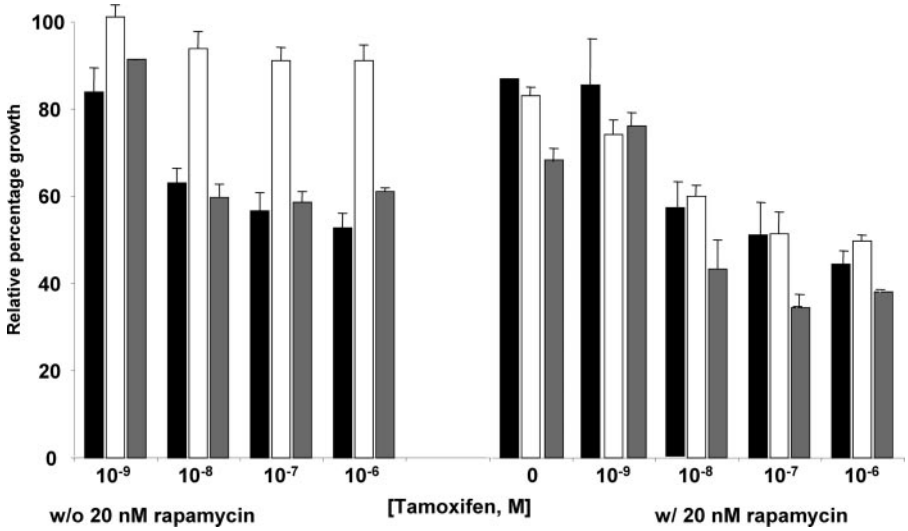


Fig. 2 Proliferation response to *in vitro* tamoxifen and rapamycin treatment. Control (■), myrAkt1 clone 13 (□), and myrAkt1 clone 29 () MCF-7 cells were grown for 96 hours in the presence of increasing concentrations of tamoxifen (0, 10^{-9} to 10^{-6}), either alone (*left panel*), or with 20 nmol/L rapamycin (*right panel*). Growth was assessed by MTT dye conversion and presented as the percentage of cell growth compared with vehicle-treated cells grown in the same culture plate at 96 hours. The data presented were the average of four replicate experiments.

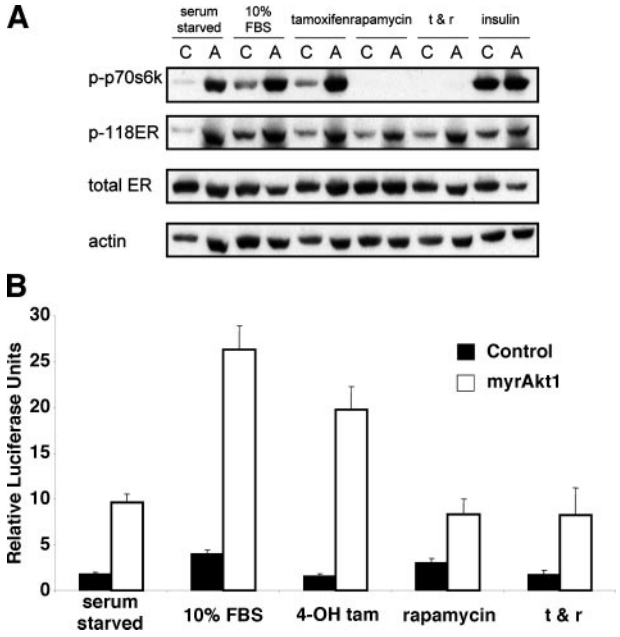


Fig. 3 Rapamycin effects on ER-α. *A*, protein lysates from control (C) and myrAkt1 (A) MCF-7 cells that had been serum starved or placed in 10% FBS-containing media with vehicle (10% FBS), 10^{-7} mol/L tamoxifen, 20 nmol/L rapamycin, both tamoxifen and rapamycin, or 100 nmol/L insulin were subjected to immunodetection for expression of phosphorylated p70s6k, phosphorylated ER-α at the 118 serine residue, total ER-α, or actin as a loading control. *B*, ER-α activity was assessed in the control (■) and myrAkt1 (□) cells with an ERE-tk-luciferase reporter. Activity was assessed under unstimulated (serum starved) or stimulated (10% FBS) conditions, as well as stimulated conditions with 10^{-7} mol/L tamoxifen, 20 nmol/L rapamycin, or a combination of tamoxifen and rapamycin. Results are the combination of three independent experiments done in triplicate and are presented as luciferase units normalized to Renilla.

starved) and stimulated (10% FBS) conditions. In addition, we assessed the effects that tamoxifen and rapamycin treatment would have on ER-α–transcriptional activity under stimulated conditions. Under unstimulated conditions, ER-α–transcriptional activity was approximately four times greater in the myrAkt1 MCF-7 cells (Fig. 3*B*, white bars) compared with the control cells (Fig. 3*B*, black bars). Stimulation with 10% FBS resulted in a 2-fold increase in activity in the control cells and ~2.5-fold increase in the myrAkt1 cells. Treatment of cells in 10% FBS with 10^{-7} mol/L tamoxifen resulted in a decrease in activity in the control cells by 59%. ER-α activity in the myrAkt1 cells was reduced 25%. Interestingly, treatment with 20 nmol/L rapamycin resulted in a decrease in activity in the control cells by only 25% but by 68% in the myrAkt1 cells. The combination of tamoxifen and rapamycin resulted in a decrease of ER-α activity by 56 and 69% in the control and myrAkt1 cells, respectively.

These data suggest that rapamycin may effectively modulate ER-α–transcriptional activity.

CCI-779 Inhibits mTOR Activity and Restores Tamoxifen Response in Refractory Breast Xenografts. To evaluate the effects of mTOR inhibition on tamoxifen response *in vivo*, we implanted xenografts of the control and myrAkt1 clone 13 MCF-7 cells into contralateral flanks of female nude mice that had been implanted s.c with a 17β-estradiol biodegradable 90-day sustained-release pellet (1.7 mg of estradiol per pellet) 10 days before tumor cell implantation. We primed with estrogen because previous studies in our lab demonstrated that although the myrAkt1 MCF-7 cells were able to establish tumors and proliferate without priming, the control MCF-7 cells were not (data not shown), suggesting that the expression of the constitutively active Akt facilitates hormone-independent growth. Tumors were allowed to grow to a size of 200 mg, at which point, animals were sorted into four (4) treatment groups containing ten (10) animals each treated with either: vehicle alone, 500 μg tamoxifen/animal s.c., five times per week, 10 mg/kg CCI-779 i.p. five times per first week only, or a cotreatment with tamoxifen and 10 mg/kg CCI-779 (CCI-779 administered for the first week only but the tamoxifen continued for the course of the experiment). This is a standard treatment protocol for tamoxifen and one that we found to be successful for CCI-779 in previously published studies (13). Treatment continued for either 24 hours or until any of the tumors reached a volume of 1 g (day 17; Fig. 4).

Both control and myrAkt1 tumors were harvested after 24 hours of treatment from five (5) mice in each treatment group. Protein lysates were obtained from each tumor and assessed for expression levels of phospho and total Akt, p70s6k, 4EBP1, the ER, and actin as a loading control (Fig. 4). As observed previously in tumor cell lines with high Akt activity (19), we found that in the control-treated myrAkt1 tumors the phosphorylation state of p70s6k was markedly elevated in comparison to that observed in the Control tumors. p70s6k phosphorylation was inhibited in CCI-779–treated mice. Interestingly, CCI-779 was more effective at blocking phosphorylation of p70s6k in the myrAkt1 tumors. This correlates with both *in vitro*, as well as *in vivo* data relating a greater sensitivity to mTOR inhibitors in cell types displaying aberrant Akt activity due to loss of PTEN (20). We examined this issue additionally by characterizing the status of 4EBP1, an Akt/mTOR-regulated protein that blocks translation of 5′-cap mRNAs by binding the initiation factor eIF4E (21). The level of hyperphosphorylated 4EBP1 was elevated in the myrAkt1 tumors relative to control tumors. Treatment with CCI-779 caused a decrease in hyperphosphorylated 4EBP1 in both control, as well as myrAkt1 tumors (to a greater degree in the myrAkt1 tumors, as with the p70s6k), indicating that CCI-779 did indeed diminish mTOR activity in both the control, as well as myrAkt1-treated tumors. Surprisingly, tamoxifen was also able to diminish phosphorylated levels of p70s6k, as well as 4EBP1 in the control tumors, but to a lesser extent than CCI-779. A full explanation of this result requires additional analysis. Neither tamoxifen nor CCI-779 had any significant effect on expression levels of either phosphorylated or total Akt or the ER. Total protein levels remained unchanged by any of the treatments.

Both control and myrAkt1 tumor volumes were assessed at baseline (the day that drug was first administered) and then on days 3, 8, 10, and 15 by caliper measurement and then harvested on day 17 after measurement (Fig. 5*A*). As seen in Fig. 5*A*, top panel, the control tumors (solid lines) demonstrated significant tumor growth inhibition in mice treated with tamoxifen (solid triangle) or the combination of tamoxifen with CCI-779 (Fig. 5*A*, bottom panel, solid diamond), when compared with the growth of the control-treated tumors (solid square). Control

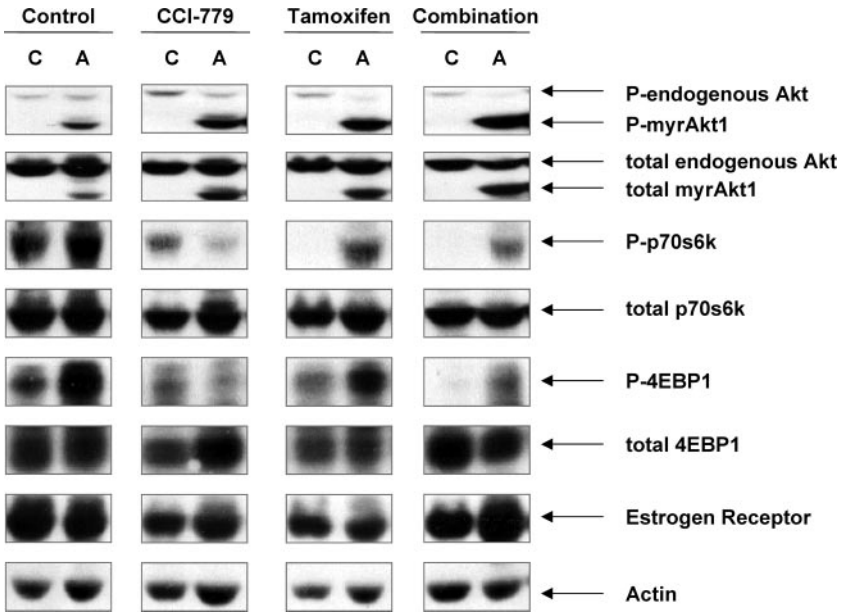


Fig. 4 CCI-779 modulation of mTOR activity *in vivo*. Control (C) and clone 13 myrAkt1 (A) xenografts were harvested from nude mice 24 hours after treatment with vehicle alone (control), CCI-779, tamoxifen, or a combination of CCI-779 and tamoxifen (combination). Protein lysates from the tumors were subjected to immunodetection with antibodies to the phospho-Ser^{473} residue of Akt on both the endogenous (*p*-endogenous Akt), as well as the myrAkt1 transgene product (*p*-myrAkt1), total Akt, phosphorylated and total p70s6k (*p*- and total p70s6k, respectively), phosphorylated and total 4EBP1 (*p*- and total 4EBP1, respectively), the ER and actin. Figures presented are representative of at least three tumors from each treatment group per cell line.

Table 1 Flow cytometry analysis of control and myrAkt1 MCF-7 cells exposed to different treatments

	Control				myrAkt1			
	Control	Rapa (20 nmol/L)	Tam (10^{-7})	Rapa + Tam	Control	Rapa (20 nmol/L)	Tam (10^{-7})	Rapa + Tam
G_1	27 ± 2 *	23 ± 3	25 ± 2	25 ± 1	45 ± 4	46 ± 3	44 ± 1	41 ± 2
S	54 ± 3	57 ± 2	57 ± 1	52 ± 2	36 ± 1	37 ± 3	40 ± 3	39 ± 2
G_2	18 ± 3	20 ± 1	18 ± 2	23 ± 3	19 ± 3	17 ± 1	16 ± 2	20 ± 1
Sub-G_1	18 ± 2	23 ± 3	35 ± 3	39 ± 3	16 ± 2	15 ± 1	14 ± 3	26 ± 1

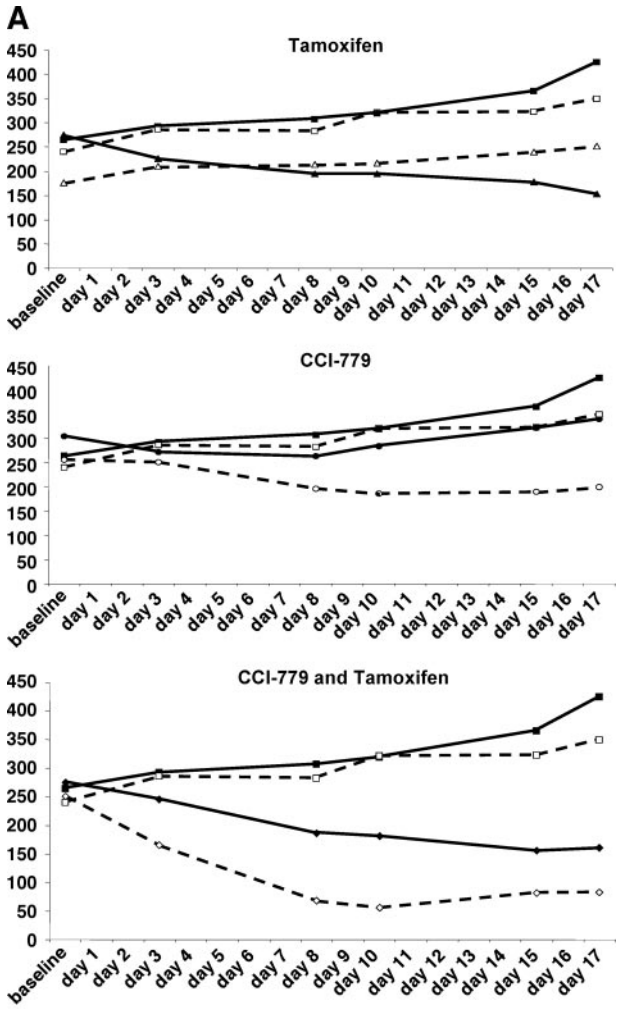
NOTE. Control and myrAkt1 MCF-7 cells were exposed for 24 hours to either vehicle (control), 20 nmol/L rapamycin (Rapa), 10^{-7} mol/L tamoxifen (Tam) or the combination of the two, then assessed for cell cycle status, as well as apoptotic (sub-G_1) status.

* Each value represents the average ± SD of three independent observations.

tumor response to CCI-779 as a single agent (Fig. 5*A*, middle panel, solid circle) was not statistically relevant. The myrAkt1 tumors (dashed line) remained resistant to the inhibitory effects of tamoxifen (Fig. 5*A*, top panel, open triangle) when administered as a single agent. The myrAkt1 tumors demonstrated a trend toward response to CCI-779 as a single agent (Fig. 5*A*, middle panel, open circle), but again this did not reach statistical significance. However, when tamoxifen was administered in combination with CCI-779 (Fig. 5*A*, bottom panel, open diamond), the myrAkt1 tumors demonstrated a considerable growth inhibitory response by day 3 of treatment.

In Fig. 5*B*, the averages and SDs of the tumor volumes on day 17, when the tumors were harvested, are presented, and the average percentage of inhibition of tumor growth is summarized in the table in Fig. 5*C*. Although both the control and the myrAkt1 tumors demonstrated some growth inhibitory response to CCI-779 treatment alone (21 and 39%, respectively, compared with the control treated tumors), these were not statistically significant. The control tumors did display significant growth inhibition to tamoxifen (64%), whereas the myrAkt1 tumors remained refractory to tamoxifen treatment (18%, not statistically significant). When tamoxifen and CCI-779 were combined, the control tumors did not display any greater inhibitory response than that observed with tamoxifen alone (62% compared with 64%). This was in contrast to the results obtained with the myrAkt1 tumors. When animals were treated with the combination of tamoxifen and CCI-779, the myrAkt1 tumors demonstrated a 76% inhibition of growth. These data would suggest that in hormone-refractory tumors with high Akt activity, CCI-779 as a cotreatment agent with tamoxifen might be effective at restoring response to these more aggressive tumors.

To determine whether the growth inhibitory results obtained from the *in vitro* MTT studies, as well as the *in vivo* xenograft studies, were due to induction of cell cycle arrest, apoptosis, or a combination of the two, we performed flow cytometry analysis on control and myrAkt1 MCF-7 cells exposed to vehicle, tamoxifen (10^{-7} mol/L), rapamycin (20 nmol/L), or a combination of the two. As seen in Table 1, none of the treatments had a significant effect on cell cycle status in either the control or myrAkt1 MCF-7 cells. Similarly, treatment with rapamycin as a single agent had little effect on the sub-G_1 (apoptotic) population in either the control or myrAkt1 cells. However, in the control cells, treatment with tamoxifen almost doubled the percent of cells observed in apoptosis from 18 to 35%. This is in contrast with the results obtained with the myrAkt1 MCF-7 cells in which exposure to tamoxifen had no



C

	Control MCF-7 Tumors	myrAkt1 MCF-7 Tumors
CCI-779	21%	39%
Tamoxifen	64%*	18%
CCI-779 and Tamoxifen	62%*	76% #

effect on apoptosis. The combination of rapamycin with tamoxifen did not enhance the apoptotic effect in the control cells (35% with tamoxifen alone compared with 36% with the combination), but significantly, in the myrAkt1 cells, the combination of rapamycin with tamoxifen increased the percent of apoptotic cells by 63% (from 16 to 26%). These data suggest that the growth inhibition that was observed in the myrAkt1 MCF-7 cells by the combination of rapamycin with tamoxifen in Fig. 2 was due, at least in part, by restoration of an apoptotic response to tamoxifen as is observed in the control cells.

We extended these *in vitro* studies to the xenograft tumors that were obtained from the CCI-779/tamoxifen animal studies. Terminal deoxynucleotidyl transferase-mediated nick end labeling analysis of the control (Fig. 6*A–D*) and myrAkt1 (Fig. 6*E–H*) tumors from the animals on control (Fig. 6, *A* and *E*), CCI-779 (Fig. 6, *B* and *F*), tamoxifen (Fig. 6, *C* and *G*), and combination (Fig. 6, *D* and *H*) treatment reveals that CCI-779 did not increase the observed average percentage of cells that were apoptotic in either the control or the myrAkt1 tumors (7.5 and 16.5%, respectively), compared with that observed in the control treatment tumors (6.5 and 13.3%, respectively). Tamoxifen treatment increased apoptosis in the control tumors to an average of 16.5% but only to 14.3% in the myrAkt1 tumors. The combination of CCI-779 with tamoxifen did not increase the average detectable percentage of cells in apoptosis in the control cells (12.1%) compared with that observed with tamoxifen as a single agent. Significantly, the combination of CCI-779 with tamoxifen increased the observed average percentage of cells in apoptosis to by 69%, to 22%. These findings are in agreement with the *in vitro* analyses and are additional indications that mTOR inhibition in the aberrant Akt setting restores normal apoptotic response to hormone therapy.

DISCUSSION

One of the principal factors explaining why current treatment with tamoxifen fails in the majority of patients with ER-positive breast cancer is the development of drug resistance, either primarily or acquired. Mechanistically, multiple factors have been implicated in the generation of tamoxifen resistance, including the anomalous activation of the PI3k/Akt signaling pathway. Previous work done by Campbell *et al.* (4) and Martin *et al.* (22), as well as studies conducted out of Carlos Arteaga's

Fig. 5 *In vivo* growth responses to tamoxifen and CCI-779. *A*. Control (*solid line*) and myrAkt1 (*dashed line*) xenograft tumor growth was recorded the day 17 of drug treatment initiation (baseline) and on days 3, 8, 10, 15, and 17. Growth of tumors in mice treated with vehicle (*squares*) were compared to those in mice treated with tamoxifen (*top panel*, *triangles*), CCI-779 (*middle panel*, *circles*) and the combination of CCI-779 and tamoxifen (*bottom panel*, *diamonds*) *B*. Control (*solid line*) and myrAkt1 (*dashed line*) xenografts were collected from nude mouse hosts after day 17 of treatment with vehicle alone (*Control squares*), tamoxifen (*triangles*), CCI-779 (*circles*), or a combination of tamoxifen and CCI-779 (*Combination*, *diamond*), and assessed for tumor volume. The data are presented as the mean and the SEM. *C*. Tumor growth inhibition on each treatment (CCI-779, tamoxifen, and the combination of CCI-779 and tamoxifen) was calculated as: 100% − (the percentage of tumor growth on treatment compared to vehicle-treated tumors).

lab (18, 23), suggest that one mechanism by which Akt confers tamoxifen resistance is through ligand-independent activation of the ER. In agreement with these studies, we have also found that ER-α–transcriptional activity, as measured by an ERE-tk-luciferase reporter assay, is higher in the myrAkt1 MCF-7 cells as compared with the control cells. We also found that treatment with tamoxifen significantly inhibits estrogen-induced activity of this reporter in the control cells but not in the myrAkt1 cells, and treatment with the PI3k inhibitor LY294002 inhibits ER-α activity in the myrAkt1 cells to levels lower than those observed in cells grown in charcoal-stripped serum without 17β-estradiol supplementation (data not shown), all in agreement with the hypothesis that Akt activates ER-α in a ligand-independent manner. However, high levels of Akt are associated with resistance to many ER-α–independent forms of breast cancer treatment, including doxorubicin, paclitaxel, and radiotherapy (5, 24). In fact, hyperactivation of this pathway has been related, in a broader fashion, to the development of resistance to multiple chemotherapy agents in many cancer types and tissues. Recent studies in a lymphoma model have shown that Akt promotes tumorigenesis and drug resistance by disrupting apoptosis (25). This suggests that Akt-mediated resistance in breast cancer is probably due to ER-α–dependent, as well as by ER-α–independent, mechanisms.

Prior studies, some of them conducted by our group (26), have demonstrated that tumors with activation of the PI3k/Akt signaling pathway, such as those with mutations in the PTEN tumor suppressor gene, are particularly susceptible to the antiproliferative effects of rapamycin, an inhibitor of the downstream target of Akt, mTOR. In addition, studies have shown that pharmacological inhibition of mTOR restores the susceptibility of PTEN-negative/Akt-positive prostate cancer cell lines to chemotherapy (13). This current study evaluated the effects of mTOR inhibition on Akt-induced tamoxifen resistance. Using a syngeneic pair of breast cancer cell lines differing only in their Akt expression levels, the results of this study demonstrate that inhibition of mTOR restores the susceptibility of Akt overexpressing breast cancer cell lines to tamoxifen. These findings are in agreement with recent studies out of Scott Lowe's laboratory (25) that showed that Akt-induced resistance to doxorubicin in a murine lymphoma model could be reversed by cotreatment with rapamycin. These data have significant mechanistic and clinical implications.

The finding that mTOR inhibition restores the susceptibility of Akt-overexpressing breast cancer cells to tamoxifen implies that mTOR might be an important mediator of survival in Akt-induced tamoxifen resistance. In fact, there is a growing body of evidence that mTOR inhibition potentates tumor response to many apoptotic and growth inhibitory agents (25, 27, 28). The mechanisms by which this is mediated remain unclear. Our *in vitro* proliferation and *in vivo* tumor data suggest that rapamycin and tamoxifen have an additive, if not synergistic, effect on growth in the cells expressing a constitutively active Akt. Our Western blot analyses and luciferase assays suggest that rapamycin as a single agent effectively inhibits ER-α; however the combination of rapamycin and tamoxifen did not result in a synergistic or even additive inhibition of ER-α in the myrAkt1 cells, suggesting that other mTOR targets contribute to the observed *in vitro* and *in vivo* growth inhibitory responses to the combination of tamoxifen with rapamycin.

In addition to p70s6k and 4EBP1, mTOR is known to regulate the oncogenic cMyc (29), as well as hypoxia-inducible factor 1α (30). Significantly, recent studies by Huang *et al.* (31) suggest that 4EBP1 activity is especially relevant for rapamycin-induced apoptosis. Our xenografts from animals treated with CCI-779 either as a single agent or in combination with tamoxifen demonstrated significant inhibition of 4EBP phosphorylation. Studies are currently ongoing in our lab to ascertain the exact mechanisms by which mTOR inhibition restores tamoxifen response, and whether this indicates that Akt mediates tamoxifen resistance through both ER-regulated as well as non-ER mechanisms.

The results from this study also have significant implications in the design of clinical studies with mTOR inhibitors in patients with breast cancer. As mentioned before, the ester of rapamycin, CCI-779, is currently being developed for the treatment of patients with cancer. Preliminary results from a randomized phase II study of CCI-779 in patients with refractory breast cancer has yielded a 15% response rate in patients with advanced disease and additional studies are planned in this disease (12). Potential strategies for additional development

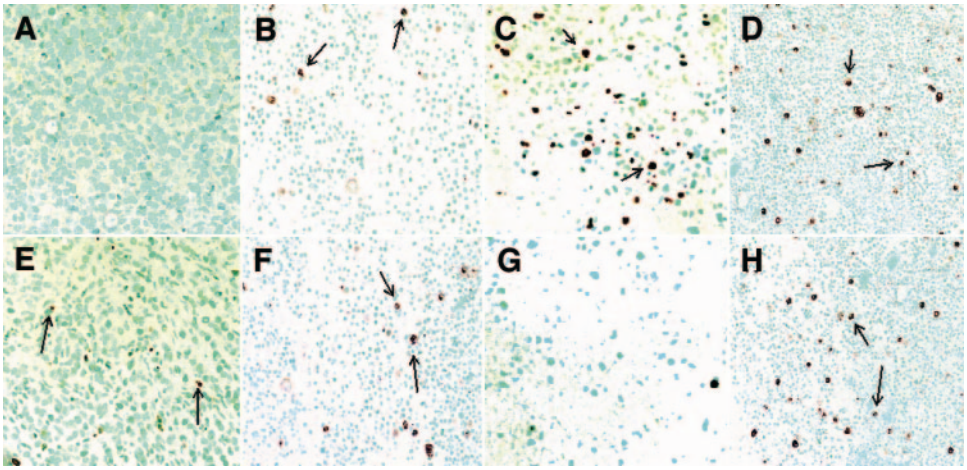


Fig. 6 *In vivo* apoptosis assessment. Control (*top panels, A–D*) and myrAkt1 (*bottom panel, E–H*) tumors from animals treated with vehicle (*A* and *E*), CCI-779 (*B* and *F*), tamoxifen (*C* and *G*), or the combination of CCI-799 with tamoxifen (*D* and *H*) were harvested after 17 days treatment, processed for histology, and analyzed for terminal deoxynucleotidyl transferase-mediated nick end labeling staining. Positive terminal deoxynucleotidyl transferase-mediated nick end labeling staining is highlighted with *arrows*. Magnification, ×200.

include phase I/II studies in combination with standard agents followed by phase III studies testing whether the combination is superior to the standard agent alone or phase III studies of single agent CCI-779 *versus* standard treatment in patients with refractory disease. Another approach, based on the results of this study, is the selection of patients with ER-positive, Akt-overexpressing tumors who are resistant to tamoxifen, either primarily or after a period of treatment for continued treatment with tamoxifen plus CCI-779. The availability of immunohistochemical methods to measure *p*-Akt overexpression in paraffin-embedded tissues makes the selection criteria for such a study straightforward. This strategy has been previously used in the clinical development of inhibitors of the epidermal growth factor receptor and, although seriously criticized for methodological questions, if properly conducted, remains an attractive strategy to explore this hypothesis in the clinic.

In conclusion, our studies found that high levels of Akt activity correlated with hormone-independent growth, increased mTOR-signaling activity, and resistance to tamoxifen therapy. Significantly, treatment with the mTOR inhibitor, rapamycin, or its analogue, CCI-779, which is currently in clinical trials, restores tamoxifen response in ER-α–positive breast cancer cells with high Akt activity. These data have important implications with regard to the mechanism of Akt induced tamoxifen resistance and the development of mTOR inhibitors in breast cancer. Our results demonstrate that the combination of rapamycin and tamoxifen has excellent anticancer activity in preclinical models of Akt-induced tamoxifen resistance and therefore may have clinical utility in treating resistant breast tumors.

ACKNOWLEDGMENTS

We thank Letitia Fulcher, Josep-Maria Peralba, and James Freeman for their invaluable technical and intellectual assistance.

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Clin Cancer Res 2004;10:8059-8067.