Determinants of Rapamycin Sensitivity in Breast Cancer Cells

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ABSTRACT

Purpose: Rapamycin inhibits the serine-threonine kinase mammalian target of rapamycin (mTOR), blocking phosphorylation of p70 S6 kinase (S6K1) and 4E-binding protein 1 (4E-BP1) and inhibiting protein translation and cell cycle progression. Rapamycin and its analogues are currently being tested in clinical trials as novel-targeted anticancer agents. Although rapamycin analogues show activity in clinical trials, only some of the treated patients respond. The purpose of this study is to identify determinants of rapamycin sensitivity that may assist the selection of appropriate patients for therapy.

Experimental Design: Breast cancer cell lines representing a spectrum of aberrations in the mTOR signaling pathway were tested for rapamycin sensitivity. The expression and phosphorylation state of multiple components of the pathway were tested by Western blot analysis, in the presence and absence of rapamycin.

Results: Cell proliferation was significantly inhibited in response to rapamycin in 12 of 15 breast cancer cell lines. The ratio of total protein levels of 4E-BP1 to its binding partner eukaryotic initiation factor 4E did not predict rapamycin sensitivity. In contrast, overexpression of S6K1, and phosphorylated Akt independent of phosphatase and tensin homologue deleted from chromosome 10 status, were associated with rapamycin sensitivity. Targeting S6K1 and Akt with small interfering RNA and dominant-negative constructs, respectively, decreased rapamycin sensitivity. Rapamycin inhibited the phosphorylation of S6K1, ribosomal S6 protein, and 4E-BP1 in rapamycin-resistant as well as -sensitive cells, indicating that its ability to inhibit the mTOR pathway is not sufficient to confer sensitivity to rapamycin.

In contrast, rapamycin treatment was associated with decreased cyclin D1 levels in the rapamycin-sensitive cells but not in rapamycin-resistant cells.

Conclusions: Overexpression of S6K1 and expression of phosphorylated Akt should be evaluated as predictors of rapamycin sensitivity in breast cancer patients. Furthermore, changes in cyclin D1 levels provide a potential pharmacodynamic marker of response to rapamycin.

INTRODUCTION

Rapamycin, a macrolide fungicide, was first isolated from Streptomyces hygroscopicus in the early 1970s and initially developed clinically for its immunosuppressant properties. Subsequently, rapamycin became of significant interest as a potential anticancer drug. Rapamycin inhibits the serine threonine kinase mammalian target of rapamycin (mTOR) by binding to one of the immunophilin family of FK 506-binding proteins, FKBP 12 (1, 2). The inhibition of mTOR decreases the phosphorylation and activation of S6K1 and 4E-binding protein 1 (4E-BP1), and this in turn inhibits the translation of critical mRNAs that are involved in the cell cycle progression and cell proliferation that are hallmarks of carcinogenesis (1, 2). Clinically, rapamycin analogues with improved stability and pharmacological properties have been well tolerated by patients in Phase I trials, and the agents have shown a promising antitumor effect in several types of refractory tumors, including breast cancer (3–5). However, only a minority of patients in each tumor lineage appear to respond to rapamycin analogues. Thus, there is an urgent need to identify markers of rapamycin sensitivity to allow prospective selection of patients likely to respond to rapamycin analogues in clinical trials.

mTOR (also known as RAFT1, RAPT1, and FRAP) modulates at least two separate downstream pathways that are conjectured to control the translation of specific subsets of mRNAs (6). In one pathway, mTOR directly phosphorylates eukaryotic initiation factor 4E-BP1, which triggers additional phosphorylation events that cause hyperphosphorylation of 4E-BP1 to dissociate from eukaryotic initiation factor 4E (eIF4E), thereby increasing the availability of functional eIF4E. eIF4E is thought to be the rate-limiting component for cap-dependent translation. The increase in free eIF4E levels thus leads to more efficient cap-dependent translation initiation, increasing the translation of mRNAs with long, highly structured 5′-untranslated regions, such as cyclin D1 and c-myc (7, 8). The second downstream target of mTOR is S6K1. It is unclear whether mTOR activates S6K1 by direct phosphorylation or the inhibition of a phosphatase (9). Regardless, on phosphorylation/activation by mTOR, S6K1 phosphorylates the 40S ribosomal protein S6. This phosphorylation of the S6 protein enhances the translation of mRNAs with a 5′ terminal oligopyrimidine tract, such as elongation factor-1α and ribosomal proteins (10).

S6K1 and 4E-BP1 are also regulated by phosphorylidyinositol 3′-kinase (PI3-K) and its downstream target Akt. In vitro,
Akt can phosphorylate mTOR on Ser2448, and this site is also phosphorylated on Akt activation in vivo (11, 12). However, although mTOR appears to be directly downstream of PI3-K/Akt in a linear pathway, Akt may also regulate mTOR in a more complex fashion, because mTOR is inhibited by the tuberous sclerosis gene products TSC1 and TSC2, which are also targets for Akt (13). S6K1 is activated by several different stimuli as a result of phosphorylation of multiple different sites. The PI3-K inhibitors Wortmannin and rapamycin, however, cause a decrease in S6K1 phosphorylation, particularly at phosphorylation sites shown to be dependent on the PI3-K pathway (14). Interestingly, a Δ2–46/ΔCT104 mutant of S6K1 is inhibited by Wortmannin but not rapamycin (15), suggesting that the PI3-K pathway can exhibit effects on S6K1 independent of regulation of mTOR activity.

The PI3-K/Akt/mTOR signaling pathway is regulated by the tumor suppressor gene product phosphatase and tensin homologue deleted from chromosome 10 (PTEN; Ref. 16). Germ-line mutations in the PTEN tumor suppressor gene lead to abnormal activation of the PI3-K/Akt pathway. Germ-line mutations in PTEN are responsible for Cowden’s syndrome, which predisposes to breast cancer (17). Although PTEN mutations are rare in sporadic cases of breast cancer, PTEN is at a site of frequent allelic imbalance, and the PTEN protein is absent or decreased in a significant number of breast cancers (16, 18, 19).

Increased signaling through the PI3-K pathway as a consequence of deletion of PTEN has been proposed as an indicator of sensitivity of rapamycin (2, 20, 21). However, Yu et al. (22) reported that, of eight breast cancer cell lines they tested, two were PTEN deficient, but six were sensitive to rapamycin analogue CCI-779. This suggests that sensitivity to mTOR inhibitors is not limited to PTEN-deficient breast cancers. The genetic and molecular abnormalities that render cells sensitive to rapamycin, particularly in breast cancer, remain unclear.

Here, we report an in vitro study of the effects of rapamycin in a panel of breast cancer cell lines representing a spectrum of aberrations in the PI3-K mTOR pathway. The results show that rapamycin induces G1 cell cycle arrest of breast cancer cell lines expressing high levels of phospho-Akt, irrespective of their PTEN status, as well as cells highly expressing phospho-S6K1. Targeting Akt and S6K1, with dominant-negative constructs and small interfering RNA, respectively, decreases rapamycin sensitivity. Our results demonstrate that phospho-Akt and overexpression of S6K1 confer rapamycin sensitivity and may be useful predictive markers of sensitivity. Furthermore, our results suggested that cyclin D1 may be useful as a pharmacodynamic marker of response to rapamycin and its analogues in breast cancer patients.

MATERIALS AND METHODS

Cell Lines and Cultures. The following human breast cancer cell lines were obtained from the American Type Culture Collection: MCF-7, BT-20, BT-549, SK-BR-3, MDA-MB-231, MDA-MB-361, MDA-MB-468, BT-474, BT-483, T-47D, MDA-MB-453, and ZR-75-1. MDA-MB-435 and MDA-MB-330 were obtained from the M. D. Anderson Breast Cancer Research Program Core Laboratory Cell Line Depository. The NCI/ADR-RES cells were obtained from the National Cancer Institute Division of Cancer Treatment and Diagnosis Tumor Repository. The HER2/neu-transformed NIH3T3 (HER2/neu-3T3), DN-Akt (kinase-dead) transfectants of the HER2/neu-3T3 cells, and DN-Akt transfectants of MDA-MB-453 cells have been described previously (23). All cell lines were cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1% penicillin–streptomycin at 37°C and humidified 5% CO2.

Reagents. Rapamycin and antibodies against phospho-Akt (Ser473), mTOR, phospho-mTOR (Ser2448), phospho S6K1 (Thr389), total S6K1, phospho-S6 ribosomal protein (Ser235/236), phospho-4E-BP1 (Ser65, Thr46), phospho-eIF2α (Ser51) were purchased from Cell Signal Technology, Inc. (Beverly, MA). Antibodies against PTEN, total 4E-BP1, and eIF4E were purchased from Cascade Bioscience (Winchester, MA), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and BD Bioscience (San Jose, CA), respectively. Antibodies against cyclin D1, eEF-1α, and c-myc were obtained from NeoMarkers, Inc. (Fremont, CA), Upstate Biotechnology (Waltham, MA), and Oncogene Research Products (San Diego, CA), respectively. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Treatment and Cell Proliferation Assays. To test the effect of rapamycin on cell proliferation, cells were plated into 96-well, flat-bottomed plates at 2–4 × 103 cells/100 μl well, with the density determined on the basis of the growth characteristics of each cell line. After the overnight incubation, triplicate wells were treated with varying concentrations of rapamycin ranging from 1 to 100 nM for 4 days. Relative percentage of metabolically active cells relative to untreated controls was then determined on the basis of the mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazane. The amount of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide that is converted to formazane indicates the number of viable cells. The results were assessed in a 96-well format plate reader by measuring the absorbance at a wavelength of 540 nm (A540 nm). The percentage of metabolically active cells was compared with the percentage of control cells growing in the absence of rapamycin in the same culture plate. The IC50s were determined by nonlinear regression analysis using the equation for a sigmoid plot. The rates of DNA synthesis were determined by the percentage of cells showing [3H]thymidine incorporation into DNA. In brief, after the cells were treated with rapamycin in the same manner as in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, 0.5 μCi of [3H] thymidine was added to each well, and the cells were incubated for an additional 16 h before being harvested. The incorporation of [3H] thymidine was measured by liquid scintillation counting. The rates of DNA synthesis in the treated cells were compared with the rates seen for control cells not treated with rapamycin in the same culture plate.

Cell Cycle Analysis and Determination of Apoptotic Cells. Cells were incubated with or without 100 nM rapamycin for 4 days and harvested when they reached a confluency of 50–70%. After the cells were washed with PBS, they were fixed with 75% ethanol overnight at 4°C. The cells were then washed twice with PBS and resuspended in hypotonic propidium iodide solution (10 μg of propidium iodide, 10 μg of RNase A, and 0.5% Tween 20 in 1 ml of PBS) for 1 h at room temperature and
kept in the dark at 4°C before analysis. Cell cycle distribution was determined by analyzing 1000–20,000 cells using a FACScan flow cytometer and Cell Quest software (Becton Dickinson, San Jose, CA). The percentage of apoptotic cells was determined by the subG0 peak in the DNA histogram.

Western Blot Analysis. Cultured cells were washed with cold PBS and lysed in lysis buffer as described elsewhere (15). To test the effect of rapamycin on the expression of mTOR and its downstream molecules, cells were treated with 100 nM rapamycin for 24 h before lysis. Cell lysates containing 50 μg of protein were separated by SDS-PAGE with 7–12.5% gel, depending on their molecular weight, and transferred to a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.02% Tween 20 and immunoblotted with antibodies, as specified in the Reagents section. The immunoblots were visualized by an enhanced chemiluminescence detection system (Amersham Life Sciences, Arlington Heights, IL).

Small Interfering RNA (siRNA). To silence S6K1, a single transfection of siRNA duplex was performed using Lipofectamine reagent according to manufacturer’s protocol (Life Technologies, Inc.). A 21-mer double stranded RNA with d(TT) overhang was selected for ability to silence S6K1 expression. The siRNA target sequence was AAUGUAUGACAUGUGTT. The siRNA was synthesized by Dharmacon Research (Lafayette, CO). Nonspecific control siRNA (Duplex X) was purchased from Dharmacon Research.

Statistical Analysis. Results were presented as means ± SD for three separate experiments. For comparison between groups, data were analyzed by Student’s t test. Differences between groups were considered statistically significant at P < 0.05.

RESULTS

Expression of mTOR and its Downstream Molecules. To determine the baseline activity of the PI3-K/Akt/mTOR signaling pathway in breast cancer cell lines, we measured the basal expression level of mTOR and related molecules by Western blot analysis (Fig. 1). The tumor suppressor gene PTEN, which negatively regulates the PI3-K/Akt/mTOR signaling pathway by counteracting PI3-K, was not detected in the BT-549 and MDA-MB-468 cell lines, consistent with previous reports of PTEN mutations in these cells (19). Phospho-Akt, a downstream component of the PI3-K pathway, was present in four of the eight cell lines, including the two PI3-K-deficient cell lines, indicating that Akt was strongly activated in these cells. The direct target of rapamycin, mTOR, and its phosphorylated/activated form was present at relatively similar levels in all eight cell lines. Indeed, there was no obvious correlation between phospho-Akt and phospho-mTOR levels in these lines.

S6K1 is one of the major downstream components of the mTOR signaling pathway. Phospho-S6K1 (Thr389) was present at high levels in MCF-7 and MDA-MB-361 cells, indicating that S6K1 was highly activated in these cells. The 40S ribosomal protein S6 is phosphorylated by activated S6K1; phospho-S6 protein (Ser235/236) was present at similar levels in all eight cell lines. Once again, phospho-S6K1 did not directly correlate with phospho-S6 protein, suggesting additional modes of regulation. In addition to S6K1, mTOR can phosphorylate 4E-BP1. We used three antibodies against 4E-BP1 to detect the total 4E-BP1 protein levels, Ser65 phospho-4E-BP1 and Thr46 phospho-4E-BP1. Although Thr46 has been shown to be a direct target site of phosphorylation by mTOR, Ser65 may be phosphorylated by additional kinases. The band intensities of total 4E-BP1 differed, but the amounts of phospho-4E-BP1 roughly correlated with those of the total 4E-BP1, indicating that the proportions of phosphorylated 4E-BP1 were similar in all cell lines. 4E-BP1 was expressed almost equally in all eight cell lines, but phospho-Akt, phospho-eIF4E and phospho-4E-BP1 were present at relatively similar levels in all cell lines. Indeed, there was no obvious correlation between phospho-Akt and phospho-mTOR levels in these lines.

In summary, phosphorylated mTOR is ubiquitously expressed. Although only two cell lines were PTEN negative, Akt was phosphorylated in four of the eight cell lines. mTOR as well as its targets, 4E-BP1 and S6K1, were phosphorylated in all eight cell lines. Thus, the mTOR signaling pathway is activated...
in all of the breast cancer cell lines tested. However, the proximal mediators of the activation of mTOR and its substrates appear to vary between the different lines.

Sensitivity of Breast Cancer Cell Lines to Rapamycin. To examine the sensitivity of each cell line to rapamycin, cells were treated with rapamycin at different concentrations for 4 days, and cell proliferation was measured by a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 2A) and DNA synthesis by thymidine incorporation (Fig. 2B). All of the cell lines, with the exception of MDA-MB-231 and MDA-MB-435, were inhibited by rapamycin in both of the assays, indicating that the decrease in cell number was accompanied by a decrease in S phase progression (thymidine incorporation). BT-20, BT-549, SK-BR-3, and MDA-MB-468 cells, which express high levels of phospho-Akt, were significantly inhibited by rapamycin. However, two cell lines, MCF-7 and MDA-MB-361 cells, which did not exhibit high levels of phosphorylated Akt, were also inhibited by rapamycin. Thus, although high Akt activity may confer sensitivity to rapamycin, it is not required for rapamycin sensitivity. Intriguingly, MCF-7 and MDA-MB-361 cells highly express phospho-S6K1 and are known to have an amplification of the S6K1 gene (26), potentially conferring sensitivity to rapamycin. MDA-MB-231 and MDA-MB-435 cell lines, which express PTEN, do not have activated Akt, and do not highly express phospho-S6K1, were resistant to growth inhibition induced by rapamycin. Thus, any of the three aberrations, high AKT phosphorylation, PTEN absence, or amplification of S6K1, is sufficient to engender sensitivity to rapamycin.

Mechanism of Rapamycin-Mediated Growth Inhibition. The decreased cell growth induced by rapamycin could be attributable to decreased cell cycle progression or increased apoptosis. As shown in Fig. 3, A and B, after 4 days of treatment with 100 nM rapamycin, the percentage of cells in the G1 phase was increased in the rapamycin-sensitive cell lines. In contrast,
the percentage of apoptotic cells in the subG\textsubscript{1} peak did not increase after treatment with the same concentration of rapamycin (Fig. 3C). These results indicated that rapamycin inhibited cell proliferation by arresting the cell cycle in the G\textsubscript{1} phase but did not induce apoptosis in the breast cancer cell lines studied under the conditions and time course analyzed.

The 4E-BP1:eIF4E Ratio As a Predictor of the Intrinsic Sensitivity to Rapamycin in Breast Cancer Cell Lines. We next sought additional molecular markers that can predict sensitivity to rapamycin in breast cancer cells on the basis of rapamycin’s mechanism of action, which is to reduce the phosphorylation of 4E-BP1, thereby enhancing the binding affinity of 4E-BP1 to eIF4E and decreasing the availability of eIF4E. On the basis of this mechanism, the ratio of the total protein levels of 4E-BP1:eIF4E has been suggested to reflect acquired or intrinsic resistance to rapamycin in rhabdomyosarcoma and colon carcinoma cells (27). This contention prompted us to examine whether the 4E-BP1:eIF4E ratios correlated with rapamycin sensitivity in breast cancer cell lines. As shown in Fig. 4, however, this ratio did not correlate with the intrinsic sensitivity to rapamycin in breast cancer cell lines.

Phospho-Akt and S6K1 As Predictors of Rapamycin Sensitivity. On the basis of our breast cancer cell line sensitivity pattern, we hypothesized that phospho-Akt and overexpression of S6K1 are predictors of rapamycin sensitivity. We therefore determined the rapamycin sensitivity of an additional panel of breast cancer cell lines to validate the efficacy of these predictive markers. We treated breast cancer cells with escalating doses of rapamycin and determined the IC\textsubscript{50}s of seven other breast cancer cell lines, BT-474, T-47D, ZR-75-1, BT-483, MDA-MB-453, MDA-MB-330, NCI/ADR-RES, as well as the rapamycin-resistant MDA-MB-231 cell line. Western blot analysis was performed to determine the expression of PTEN, phospho-Akt, phospho-S6K1, total S6K1, and actin (Fig. 5).

Six of the eight cell lines tested in this panel were sensitive to rapamycin. High levels of phospho-Akt were detected in all six rapamycin-sensitive cell lines, whereas only one cell line, BT-483, did not express PTEN. These results confirm that expression of phospho-Akt is a predictor of rapamycin sensitivity, independent of PTEN status.

Two of the cell lines, BT-474 and ZR-75-1, overexpressed total S6K1. These two cell lines were among the most rapamycin-sensitive cells within our panel. These results confirm that S6K1 overexpression is associated with rapamycin sensitivity.

Similar to MDA-MB-231 and MDA-MB-435, which are resistant to rapamycin (see Figs. 1 and 2), NCI/ADR-RES did not express high levels of phospho-AKT or phospho-S6K1 and expressed wild-type levels of PTEN. This supports the contention that the absence of all three of these aberrations correlates with resistance to rapamycin.

The Effect of Modulation of S6K1 and Akt on Rapamycin Sensitivity. To test whether S6K1 overexpression confers rapamycin sensitivity, we used siRNA oligonucleotides to silence the expression of S6K1. The siRNA sequence we selected markedly decreases total S6K1 protein levels 72 h after transfection as demonstrated in Fig. 6A. MCF-7 cells were transfected with either S6K1 siRNA or a nontargeting control siRNA. After transfection (48 h), the cells were incubated with or without rapamycin. S6K1 siRNA-transfected MCF-7 cells were less sensitive to the effects of rapamycin (Fig. 6, B and C).
To determine the effect of PI3-K/Akt signaling on rapamycin sensitivity, we compared the rapamycin sensitivity of phospho-Akt expressing cells with isogenic dominant-negative-Akt (DN-Akt, kinase-dead)-transfected cells. We found that MDA-MB-453 breast cancer cells, which are not PTEN null but overexpress HER2/neu and express phospho-Akt, were moderately more sensitive to rapamycin than DN-Akt-transfected MDA-MB-453 cells (Fig. 6D). We then compared NIH3T3 cells with an isogenic HER2/neu-transformed clone (HER2/neu-3T3), where AKT is constitutively activated, and an HER2/neu-transformed, DN-Akt-transfected clone (DN-Akt:HER2/neu-3T3). HER2/neu overexpression was associated with a moderate increase rapamycin sensitivity compared with the parental NIH3T3 cell line (Fig. 6E) compatible with the predicted effects of AKT activation by HER2/neu transfection. Strikingly, the introduction of a DN-AKT construct was associated with a significant reduction of rapamycin sensitivity (P < 0.0001; Fig. 6E). These results demonstrate that inhibition of the Akt pathway decreases rapamycin sensitivity.

The Degree of Inhibition of mTOR and its Downstream Molecules As a Predictor of Rapamycin Sensitivity. Next, in an attempt to identify pharmacodynamic characteristics that correlate with responsiveness to rapamycin, the phosphorylation of mTOR’s downstream targets after treatment with rapamycin for 24 h was evaluated using Western blot analysis. There was a slight decrease in the level of phospho-mTOR in five of the eight cell lines, but this did not correlate with their sensitivity to rapamycin-induced growth inhibition. Furthermore, phospho-p70 S6K1 levels were markedly decreased, and phospho-S6 protein levels were reduced to nearly undetectable levels in all cell lines (Fig. 7), once again independent of the effect of rapamycin on cell growth.

Because phosphorylated eIF2α is known to prevent the formation of the eIF2.GTP.Met-tRNAi tertiary complex and inhibit global protein synthesis (7), we also examined the change in phospho-eIF2α in response to rapamycin. As shown in Fig. 7, the levels of phospho-eIF2α were somewhat increased by rapamycin in the MCF-7, BT-20, and SK-BR3 cell lines, but there were no prominent changes in the phospho-eIF2α levels in...
We next evaluated the useful monitors of the effectiveness of rapamycin. It did not correlate with the extent to which phosphorylation of downstream molecules, the extent to which phosphorylation of mTOR’s targets were inhibited did not correlate with the extent to which cell growth was inhibited by rapamycin. This indicated that phospho-S6K1 and -4E-BP1 are not likely to prove to be useful monitors of the effectiveness of rapamycin. However, rapamycin reduced the level of phospho-mTOR and its downstream targets were inhibited did not correlate with the extent to which phosphorylation of mTOR’s targets were inhibited did not correlate with the extent to which cell growth was inhibited by rapamycin. This indicated that phospho-S6K1 and -4E-BP1 are not likely to prove to be useful monitors of the effectiveness of rapamycin.

Rapamycin-Induced Inhibition of Cyclin D1 Expression in Rapamycin-Sensitive Cell Lines. We next evaluated the effect of rapamycin on the expression of mRNAs that are known to be under translational control of mTOR and its downstream targets. First, we determined the protein levels of cyclin D1 and c-myc, whose translation is regulated by eIF4E, and elongation factor-1α, whose translation is thought to be regulated by S6K1. Western blot results were quantified by densitometry, and protein levels were determined after normalizing for actin levels. Baseline cyclin D1 expression level was higher in the rapamycin-sensitive cells, MCF-7 and MDA-MB-468, with ~2-fold reduction in these levels after rapamycin treatment (Fig. 8). In contrast, the baseline cyclin D1 expression level was 2-fold lower in the MDA-MB-435 and MDA-MB-231 cells, with no reduction, but rather a slight increase, in cyclin D1 expression in response to rapamycin. c-myc levels showed 26% decrease in the MCF-7 cells with treatment but were unchanged in the other cells. Rapamycin treatment was associated with ~2-fold reduction in the elongation factor-1α levels in MCF-7 cells, but the levels remained unchanged in the other cell lines after treatment. Thus, the presence of high basal levels of cyclin D1 followed by a decrease in cyclin D1 expression correlated with responsiveness to rapamycin.

To verify that the lack of rapamycin-induced cyclin D1 downregulation is a reproducible finding in rapamycin-resistant breast cancer cells, we determined the effect of rapamycin in our third rapamycin-resistant cell line NCI/ADR-RES (Fig. 8B). We found that unlike MDA-MB-231 and MDA-MB-435 cells, that NCI/ADR-RES had moderate levels of basal cyclin D1 expression. However, rapamycin treatment of this cell line did not reduce cyclin D1 levels. These results demonstrate that expression of cyclin D1 levels with rapamycin treatment may correlate with response and holds potential as a pharmacodynamic marker.

DISCUSSION

Rapamycin and its analogues are considered among the most promising drugs in the anticancer pipeline (3). Rapamycin and its analogues are considered among the most promising drugs in the anticancer pipeline (3). Rapamycin and its analogues CCI-779 (Wyeth-Ayerst Research, Collegeville, PA) and RAD001 (Novartis, Basel, Switzerland) are now being evaluated as anticancer agents in multiple clinical trials (3, 4). Thus far, in preclinical studies, human prostate cancer, small cell lung cancer, glioblastoma, T-cell leukemia, and breast cancer have been among the most sensitive cancers to rapamycin (1).

PTEN-deficient tumors are considered the best candidates for rapamycin treatment, because the PI3-K/Akt pathway is constitutively activated in these tumor cells. This was the view taken as a result of studies in PTEN+/+ and PTEN−/− mouse cells, transformed cells of PTEN−/− mice, and human prostate cancer and multiple myeloma cells with a defined PTEN status showing that the growth of PTEN-deficient cancer cells is preferentially blocked when mTOR is inhibited (2, 20–22, 28). Extrapolating from this, we reasoned that, because PTEN mutations in sporadic cases of breast cancer are not very common, with a reported incidence of 4% (18), if rapamycin was only effective in tumors with PTEN mutations, only a small portion of breast tumors might respond to rapamycin analogues. In contrast, we found that rapamycin effectively inhibited the growth of most of the breast cancer cell lines tested. Similarly, Yu et al. (22) reported that, of the eight breast cancer cell lines...
they tested, two were PTEN deficient, but six were sensitive to the rapamycin analogue CCI-779. Thus, although PTEN-deficient cells are clearly responsive to rapamycin, additional molecular aberrations can also render cells sensitive to rapamycin. Therefore, to ensure that responsive patients are offered treatment with rapamycin or its analogues, it is important to identify additional markers or characteristics that can predict the sensitivity to rapamycin in breast cancer patients.

Recently, Dilling et al. (27) reported that the 4E-BP1: eIF4E ratio was reduced in rhabdomyosarcoma cell lines with an acquired rapamycin resistance and in several colon carcinoma cell lines with an intrinsic resistance to rapamycin. They suggested that the 4E-BP1: eIF4E ratio might therefore be a determinant of rapamycin sensitivity. However, in our study, sensitivity to rapamycin did not correlate with the 4E-BP1 phosphorylation status or 4E-BP1: eIF4E ratio, suggesting that the predictive markers for rapamycin sensitivity may not be universally applicable to different types of cancer.

Our study also showed that all breast cancer cell lines with high baseline levels of phospho-Akt, irrespective of their PTEN status, were sensitive to rapamycin. This suggested that the activation status of PI3-K/Akt, irrespective of the mechanism of this activation, was associated with rapamycin sensitivity. This agreed with the finding of Neshat et al. (20) that the enhanced tumor growth caused by constitutive activation of Akt in PTEN-/+ prostate cancer cells can be reversed by CCI-779. This is an especially intriguing finding, because signal transduction pathways that are activated in breast cancer, such as the Her2/neu pathway, activate Akt (23, 29). Supporting this hypothesis, we have demonstrated that HER2/neu overexpression in NIH3T3 cells enhances rapamycin sensitivity, with a significant reduction in rapamycin sensitivity in DN-Akt-transfected cells. Our results are consistent with those of Hermanto et al. (30) who found that the forced expression of HER2/neu in MDA-MB-231 cells sensitized the cells to inhibition of colony formation by rapamycin. These results taken together suggest that activation of the PI3-K/Akt pathway, regardless of mechanism (i.e., PTEN loss or HER2/neu overexpression), is associated with increased mTOR signaling and rapamycin sensitivity. Of particular importance, these findings also mean that many more breast tumors may respond to rapamycin analogues than would have been the case if rapamycin’s effects were limited to PTEN-null tumors.

We also observed that two cell lines, MCF-7 and MDA-MB-361, which do not have high levels of phospho-Akt but highly express phospho-S6K1, were also sensitive to rapamycin. Interestingly, both of these cell lines have a genomic amplification of the S6K1 gene located on chromosome 17q23 (31). Furthermore, the two cell lines with phospho-Akt expression that also overexpressed phospho-S6K1 and total S6K1, BT-474, and ZR-75–1 were among the most rapamycin-sensitive cell lines within our panel. Both of these cells have been shown previously to have an amplification of the S6K1 gene (32). This suggested that S6K1 amplification/overexpression could be a predictor of rapamycin sensitivity. We found that reduction in S6K1 levels mediated by siRNA leads to a decrease in rapamycin sensitivity, confirming that S6K1 overexpression is causally linked to rapamycin sensitivity. These findings are especially important because S6K1 gene is amplified in 8.8% of primary breast cancers, and the S6K1 gene is overexpressed at the RNA level in 38% of breast tumors (26, 33). Such S6K1 amplification has been associated with a poorer prognosis (26). Rapamycin analogues thus may be an especially useful molecularly targeted therapy in this subgroup of patients.

Phospholipase D-dependent accumulation of phosphatidic acid is required for activation of mTOR signaling, and it has been suggested that rapamycin competes with phosphatidic acid for the FKBP12-rapamycin-binding domain in mTOR (34). Recently, Chen et al. (35) have reported that increased phospholipase D activity predicts rapamycin resistance in breast cancer cell lines. Indirectly assessing phospholipase D activity by determining total and phosphorylated phospholipase D expression, along with assessing predictors of rapamycin sensitivity identified in our study, phospho-Akt, and S6K1, may further optimize selection of patients that will derive the most benefit from rapamycin and its analogues.

Treatment with the rapamycin analogues have been shown...
to inhibit S6K1 activity in tumors, skin, and peripheral lymphocytes in animal models (36, 37). In fact, it has been proposed that peripheral blood lymphocytes may be used as a surrogate biomarker when planning dosing regimens. Alternatively, Dudkin et al. (38) found that when 4E-BP1 phosphorylation (Thr70) was inhibited by CCI-779, this correlated with the growth inhibition of prostate, glioma, and ovarian carcinoma xenografts by CCI-779, prompting them to propose that phospho-4E-BP1 status may be useful for determining whether mTOR activity is inhibited in tumor specimens. In our study, 4E-BP1 phosphorylation and S6 phosphorylation were inhibited in both rapamycin-sensitive and -resistant cell lines. Our results are therefore similar to those from studies of prostate cancer and multiple myeloma cells (20, 28) and suggest that although these measurements may indicate that a biologically relevant dose of rapamycin is present, these will not be useful in predicting which patients will respond to the drug. Taken together, these results showed that the differential sensitivity to rapamycin is not explained by differential ability to inhibit the mTOR pathway. Thus, although phospho-S6K1 and phospho-4E-BP1 may be useful to monitor whether the drug levels are sufficiently high to achieve adequate target inhibition, phospho-S6K1 and phospho-4E-BP1 do not appear to be useful for predicting whether breast tumors will respond to the drug.

One explanation for this finding is that blockade of mTOR may potentially inhibit cell growth by mechanisms other than through its known effects on 4E-BP1 and S6K1. A second explanation is that, although the mTOR signaling pathway is inhibited in all cell lines, the effect on proliferation may be more dramatic in some cell lines, such as those with an activated PI3-K/Akt pathway, which may not only activate mTOR but may also potentiate its effects. A third explanation is that inhibition of the phospho-mTOR’s downstream targets 4E-BP1 and S6K1 has different downstream effects in specific cell lines, leading to differences in the gene expression and translational profile and thus alterations in the rapamycin-mediated growth response. Alternatively, although the mTOR pathway is activated in particular tumors, it may not be obligatory for continued cell cycle progression, with this process being mediated by alternative, rapamycin-insensitive pathways. Finally, mTOR may not be the only target of rapamycin, or S6K1 and 4E-BP1 may not be mTOR’s critical downstream effectors.

Rapamycin’s effects on cellular physiology is thought to be at least in part mediated by alterations in the translation of mRNAs important for cell growth and proliferation. It effectively inhibits S6K1 phosphorylation, which is thought to decrease the translation of 5’ terminal oligopyrimidine mRNAs, such as elongation factor-1α (10). In our experiments, although S6 phosphorylation was dramatically inhibited in all cell lines, there was only a 2-fold reduction in the elongation factor-1α protein level in MCF-7 cells and no change in the rapamycin-sensitive MDA-MB-468 cells and rapamycin-resistant cells. This finding is especially interesting in light of the recent finding of Stolovich et al. (39) that the complete inhibition of S6K1 by rapamycin in various cell lines only mildly repressed the translation of terminal oligopyrimidine mRNAs. The inhibition of terminal oligopyrimidine mRNA translation may therefore not be an important contributor to the ability of rapamycin to inhibit cell growth or alternatively the effects of rapamycin may be more prominent in cell lines highly expressing phospho-S6K1. Alternately, S6K1 inhibition may affect cell growth through additional targets.

Rapamycin’s other translational effect is through 4E-BP1. Specifically, rapamycin decreases the hyperphosphorylation of 4E-BP1, thereby increasing 4E-BP1 binding to eIF4E and in turn inhibiting cap-dependent translation. The result demonstrated that availability of eIF4E would be expected to selectively decrease the translation of mRNA with highly structured 5’ untranslated region, such as cyclin D1 and c-myc. Indeed, we found that cyclin D1 expression, which is regulated by a cap-dependent translation, was decreased in rapamycin-sensitive breast cancer cells MCF-7 and MDA-MB-468 but not in any of the rapamycin-resistant breast cancer cell lines we identified. Thus, alterations in cyclin D1 may play an important role in the cell cycle regulatory effects of rapamycin and be a valuable predictor of response to therapy. Furthermore, the higher baseline expression of cyclin D1 in the rapamycin-sensitive cells suggests that cyclin D1 plays a relatively important role in the proliferation of these cells. However, the moderate expression of cyclin D1 in rapamycin-resistant cell lines NCI/ADR-RES demonstrates that the expression of cyclin D1 alone is not sufficient to confer rapamycin-sensitivity, but rather that down-regulation of expressed cyclin D1 may be an indicator of sensitivity.

In contrast to cyclin D1, c-myc was only minimally modulated in our experiments, with only a slight decrease in the c-myc levels in MCF-7 cells with treatment but no change in the other cells. This finding may be explained by the fact that c-myc mRNA has an internal ribosome entry site (40), which may allow the c-myc mRNA to be translated although cap-mediated translation is inhibited by rapamycin. Rapamycin may also regulate cyclin D1 by additional mechanisms. In fact, Hasemolhosseini et al. reported that NIH3T3 cells rapamycin inhibits cyclin D1 expression by decreasing cyclin D1 mRNA and protein stability (41). Thus, further work is needed to determine the mechanism of cyclin D1 regulation in breast cancer cells.

Interestingly, our findings in breast cancer cells differ from those observed with rhabdomyosarcoma cells and multiple myeloma cells. In particular, Hosoi et al. (42) found that rapamycin inhibited c-myc induction by serum and that the failure of rapamycin to inhibit c-myc transcription correlated with rapamycin resistance in rhabdomyosarcoma cells. Shi et al. (28) reported that CCI-779 inhibited the expression of c-myc in CCI-sensitive myeloma cells but not CCI-resistant cells. In contrast, cyclin D1 expression was not altered in either sensitive or resistant cells. Thus, the modulation of mTOR’s downstream targets may differ in different tumor types. Alternately, cyclin D1 may not be downregulated in all rapamycin-sensitive cell lines; in that scenario downregulation of cyclin D1 would be able to predict response, but lack of downregulation would not necessarily predict resistance. Our results suggest that cyclin D1 may prove superior to c-myc as a pharmacodynamic marker of the rapamycin response in breast cancer. However, monitoring the proteomic profile rather than individual genes may provide a greater degree of predictive power.

Previous studies in other cell types have shown that the major mechanism by which rapamycin suppressed tumor cell growth was by inhibiting of cell cycle progression in the G1.
induced apoptosis in certain types of tumors (44, 45), e.g., phase (41, 43). However, it was reported that rapamycin also induces apoptosis in rhabdomyosarcoma cell lines with deficient p53 function as a consequence of continued cell cycle progression during mTOR inhibition. In addition, Shi et al. (46) found that rapamycin increased interleukin-2 deprivation-induced apoptosis in an interleukin-2-dependent mouse T-cell line. Furthermore, the reports that rapamycin enhances the cytotoxicity of chemotherapy agents (46–48) support the hypothesis that rapamycin induces or enhances apoptosis in certain tumor types or conditions. In our study, rapamycin was not sufficient to induce apoptosis of tumor cells, irrespective of their p53 status (e.g., MCF-7 is p53 wild type; MDA-MB-468 is p53 mutant), but rather inhibited tumor cell proliferation by producing G0 cell cycle arrest. However, the preliminary results of clinical trials with rapamycin analogues suggest that some of these patients indeed did have tumor regression, consistent with a cytotoxic response (5). Thus rapamycin analogues indeed may be cytotoxic in a clinical setting, and further work is needed to better select the patients that are most likely to benefit from these therapies.

REFERENCES
Determinants of Rapamycin Sensitivity in Breast Cancer Cells


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