Antitumor Activity of Rapamycin in a Transgenic Mouse Model of ErbB2-Dependent Human Breast Cancer

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Abstract

The ErbB2 (Neu) receptor tyrosine kinase is frequently overexpressed in human breast cancers, and this phenotype correlates with a poor clinical prognosis. We examined the effects of the mammalian target of rapamycin inhibitor, rapamycin, on mammary tumorigenesis in transgenic mice bearing an activated ErbB2 (NeuYD) transgene in the absence or presence of a second transgene encoding vascular endothelial growth factor (VEGF). Treatment of NeuYD or NeuYD × VEGF mice with rapamycin dramatically inhibited tumor growth accompanied by a marked decrease in tumor vascularization. Two key events that may underlie the antitumor activity of rapamycin were decreased expression of ErbB3 and inhibition of hypoxia-inducible factor-1–dependent responses to hypoxic stress. Rapamycin exposure caused only a modest inhibition of the proliferation of tumor-derived cell lines in standard monolayer cultures, but dramatically inhibited the growth of the same cells in three-dimensional cultures, due in part to the induction of apoptotic cell death. These studies underscore the therapeutic potential of mammalian target of rapamycin inhibitors in ErbB2-positive breast cancers and indicate that, relative to monolayer cultures, three-dimensional cell cultures are more predictive in vitro models for studies of the antitumor mechanisms of rapamycin and related compounds. (Cancer Res 2005; 65(12): 5325-36)

Introduction

The potent immunosuppressive agent, rapamycin (sirolimus), is clinically approved for the prevention of organ transplant rejection and restenosis (1, 2). Several rapamycin-related compounds are in phase I, II, and III clinical trials in patients with renal cancer and other malignancies (3). Studies in a variety of human cell lines have linked the antitumor activity of rapamycin to the induction of either G1 arrest (cytostasis) or apoptotic death (3). Interestingly, although the rapamycin-sensitive signaling pathway is active in all cell types, different cell lines display widely variable sensitivities to the cytostatic or cytotoxic effects of this drug (4, 5). Two alterations that seem to increase rapamycin sensitivity are deregulated signaling through the phosphoinositide 3-kinase (PI3K) pathway or loss of p53 (3). Given that both abnormalities are extremely common in transformed cells, rapamycin and related compounds may display high therapeutic indices against a wide range of human cancers.

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Materials and Methods

Transgenic mice and tumor growth assays. NeuYD × VEGF bitransgenic mice were generated by crossing MMTV-Neu<sub>alt</sub>-YD5 mice (20) with MMTV-VEGF-164 mice as described previously (22). Female animals with the appropriate genotypes were randomized into control and drug treatment groups (20 animals per group). Rapamycin was obtained from the National Cancer Institute's Developmental Therapeutics Program and was dissolved in ethanol and stored at −80°C. For dosing of animals, the stock solution was diluted with aqueous solution to give final concentrations of 4% ethanol, 5% polyethylene glycol 400, and 5% Tween 80 immediately before IP injections (23). Drug-treated mice received 0.75 mg/kg rapamycin every second day, and control animals received the drug vehicle only according to the same dosing schedule. Tumor volume was calculated from skin caliper measurements using the formula: \( \pi / 6 \times (\text{length} \times \text{width}^2) \).

Tumor cell isolation and culture. Freshly excised tumors from NeuYD mice were minced, dispersed with 0.25% trypsin, 1 mM EDTA in PBS, and plated in MEM containing 1 mM/L pyruvate, 10 ng/mL EGF, 40 ng/mL deoxycholate, 1 mM/L \( \beta \)-estradiol, 0.1 mM/L \( \beta \)-mercaptoethanol, 2×10<sup>-4</sup> M mercaptoethanol, and 5% fetal bovine serum. Proliferating cells were selected by trypsinization and serial subcultivation in complete medium (four cycles over 4-6 weeks). The bulk cell populations were then suspended, filtered through 0.45 μm nylon mesh, and cloned by limiting dilution. Clonal cell populations bearing an epithelial morphology were expanded and screened for expression of epithelial cytokeratin K8 using an anti-(\( \alpha \)-K)K8 monoclonal antibody (TROMA1, Developmental Studies Hybridoma Center, University of Iowa, Iowa City, IA). The levels of NeuYD mRNA in the clonal lines (YD A15 and YD A19) used in the present studies were determined by quantitative real-time PCR (Q-PCR; ref. 22). NeuYD RNA was present in the cell lines at levels 36% to 60% of the average found in five independent samples from the actual NeuYD tumors.

Immunoblot analyses. Excised tumors were snap frozen in liquid nitrogen and stored at −80°C. For immunoblot analyses, frozen tissue was ground with mortar and pestle, and the powdered material was suspended in sodium deoxycholate, 0.1% SDS, 50 mmol/L NaCl, 1 mmol/L EDTA (pH 7.4) containing 1% NP40, 1% sodium glycerophosphate, 0.1% sodium orthovanadate, 0.01% leupeptin, 20 mmol/L microcin. Insoluble material was removed by centrifugation, and soluble protein (100 μg) was resolved by SDS-PAGE. The blots were probed onto polyvinylidene difluoride membranes (Millipore, Billerica, MA) and probed with the following commercially prepared antibodies: α-phospho-4E-BP1, α-cyclin D1, α-hypoxia-inducible factor-1α (HIF-1α), and α-CD31 (PharMingen, San Jose, CA) and α-ErbB3, α-p27, and α-p21 (Oncogene, Cambridge, MA). Immunoreactive proteins were detected with horseradish peroxidase–conjugated protein A (Amersham, Piscataway, NJ) for rabbit antibodies or sheep α-mouse IgG-horseradish peroxidase (Amersham) for mouse monoclonal antibodies followed by incubation with the Renaissance chemiluminescence reagent (NEN, Boston, MA). Where indicated, relative protein levels were calculated by densitometric analysis of photographic films and quantification with NIH Image 1.63 software.

Immunohistochemistry. Four hours after the last dose of rapamycin or vehicle, mice were injected with 250 mg/kg 5-bromo-2-deoxyuridine (BrdUrd) and were euthanized at 2 hours postinjection. Incorporation of BrdUrd into tumor cell DNA was detected by immunostaining of tumor tissue with α-BrdUrd monoclonal antibody (BD Biosciences, San Jose, CA). Apoptotic cells were identified with the ApopTag In situ Apoptosis Detection kit (Chemicon, Temecula, CA). Blood vessels in paraffin-embedded or formaldehyde–fixed, paraffin-embedded tumors were decorated with rat α-mouse CD31 monoclonal antibody and visualized using the Vector Laboratories (Burlington, CA) ABC kit. The density of α-CD31 staining was calculated with the count/size function of Image-Pro 4.5.1. Microvascular pericytes were identified by labeling with rabbit α-NG2 proteoglycan-specific antibody (24). Tumor samples were also immunostained with α-HIF-1α polyclonal antibody (25) or α-GLUT1 polyclonal antibody (Alpha Diagnostic International, San Antonio, TX).

Vascular endothelial growth factor protein expression. Tumor tissues were snap frozen and lysed in RIPA. VEGF levels in the tumor extracts were measured by ELISA with the Quantikine kit (R&D Systems, Minneapolis, MN).

Three-dimensional cultures. NeuYD and NeuYD × VEGF tumor-derived cell lines were trypsinized and replated at low density in Petri dishes coated with a thin layer of Matrigel (BD Biosciences). The plating medium was DMEM containing 10% FCS and 2% Matrigel. Single cells were allowed to form multicellular spheroids for 4 days in culture, at which time rapamycin treatment was initiated as described in Results. Spheroid growth length and width dimensions were determined by microscopic examination with an optical micrometer, and volume was calculated according to the formula: \( \pi / 6 \times (\text{length} \times \text{width}^2) \). Spheroids were recovered from semisolid growth medium with Matrigel Cell Recovery Solution (BD Biosciences). The isolated spheroids were either trypsinized to release single cells for flow cytometric evaluation or lysed with RIPA to produce soluble proteins for immunoblot analyses.

For apoptotic death assays, cells were cultured as monolayers or as spheroids as described above. Samples were treated for 96 hours with 100 nmol/L rapamycin or with drug vehicle only. Single-cell suspensions were stained with Annexin V-FITC and propidium iodide according to the manufacturer’s suggested protocol (PharMingen) and were analyzed immediately by flow cytometry.

Cell proliferation assay. Cells were plated in 96-well plates (2,000 cells per well) in 100 μL complete culture medium. The cells were cultured overnight, and the medium was replaced with either drug-free or rapamycin-containing complete medium. The cells were cultured for 4 days, and cellular proliferation was determined with the CellTitre 96 AQueous Nonradioactive Cell Proliferation Assay kit (Promega, Madison, WI).

Caspase-3 activity. Caspase-3 activity in cell extracts was measured with a commercially available, fluorescence-based assay (Bio-Rad Laboratories, Hercules, CA). The fluorescent product generated by the protease was measured with a Molecular Devices (Sunnyvale, CA) fMax plate reader.

Quantitative real-time PCR analysis of mRNA expression in tumors. Total cellular RNA was isolated from tumors with the RNaseasy Mini kit (Qiagen, Valencia, CA), and RNA (2 μg) from each sample was reverse transcribed with an oligo(dT)<sub>18</sub> primer and the SuperScript II First-Strand Synthesis System kit (Invitrogen, Carlsbad, CA). PCR primer sequences used in the analysis are available on request. Q-PCR reactions were done with a LightCycler instrument (Mx3000P, Stratagene, La Jolla, CA) and the Brilliant SYBR Green Q-PCR Master Mix (Stratagene). Cyclophilin A mRNA was used as an internal reference control for each cDNA preparation. PCR primers were designed with the Primer 3 algorithm.4 The nucleotide sequences of the left and right primers were ACAGCAATCCCATCTTCAC and TGTGT-CATCTCCACTCTTC for mErbB3, respectively, and CACCGGTTCCTTCGAC and ATTCTGTGAAAGGAAGGA for mCPI, respectively. Specificity of the SYBR Green Q-PCR signal was monitored by melting curve analysis and by agarose gel electrophoresis analysis, confirming that each Q-PCR product was the anticipated size. Relative gene expression was analyzed with LightCycler version 3.5 software (Roche Applied Science, Indianapolis, IN). The amount of test cDNA was normalized to that of the cyclophilin A control in the same sample.

Results

Effect of rapamycin on mammary tumorigenesis. Previous studies showed that the average time from birth to the appearance of clinically detectable tumors was 111 days in NeuYD mice, whereas tumor formation in NeuYD × VEGF mice was accelerated, with an average onset of 51 days in the bitransgenic animals (20, 22). In initial experiments, we examined the effect of low-dose (0.75 mg/kg) rapamycin therapy on tumorigenesis in NeuYD mice

when drug therapy was initiated ~2 weeks (day 92) before the emergence of palpable mammary tumors. The NeuYD × VEGF mice were also treated with rapamycin before tumor appearance, with drug administration beginning on day 33 postpartum in these animals. Tumor growth was dramatically suppressed by rapamycin in the NeuYD mice (Fig. 1A); indeed, one third of the drug-treated mice contained no visually detectable tumors at the time of sacrifice (day 132 postpartum), whereas multiple tumors were readily detected in 100% of the control animals. Consistent with the more aggressive mammary tumor phenotype observed in the NeuYD × VEGF mice, "chemopreventive" therapy with rapamycin was less effective in the suppression of tumor growth in these mice.
Nonetheless, rapamycin treatment reduced total tumor mass by 75% and drastically reduced tumor growth rates in the NeuYD × VEGF animals.

Effect of rapamycin on growth of established mammary tumors. In subsequent studies, we initiated rapamycin therapy in NeuYD and NeuYD × VEGF mice after the detection of palpable mammary tumors (diameter, ~5 mm). Again, rapamycin treatment effectively blocked tumor growth and prolonged survival of NeuYD mice (Fig. 1C). In NeuYD × VEGF animals, rapamycin treatment markedly slowed but did not abrogate tumor growth. Furthermore, tumor regrowth was evident at 10 days after discontinuation of rapamycin therapy, indicating that the antitumor activity of rapamycin was reversible rather than curative (Fig. 1D). Nonetheless, at the time of sacrifice, total tumor mass in the rapamycin-treated NeuYD × VEGF mice was reduced by ~63% relative to the control animals (Fig. 1D). The drug-induced reduction in total tumor burden reflected both a decrease in the number of detectable tumors (data not shown) and a clear reduction in the average volume of those tumors that did emerge in the rapamycin-treated NeuYD × VEGF mice (Fig. 1E).

Effect of rapamycin on tumor vascularization. Earlier results indicated that rapamycin treatment disrupts the neovascularization of an orthotopically implanted tumor in mice and provided evidence that this drug effect was due, at least in part, to the inhibition of VEGF production (21). In the present studies, we examined the effects of rapamycin therapy on the vascularity of mammary tumors in NeuYD and NeuYD × VEGF mice. In tumor tissue from NeuYD × VEGF mice, VEGF is expressed at supraphysiologic levels from a synthetic transgene lacking the normal transcriptional and post-transcriptional elements that regulate endogenous VEGF expression (26). Rapamycin treatment reduced the expression of VEGF in NeuYD mammary tumors by 46% and in NeuYD × VEGF tumors by 25%. Although these decreases in VEGF expression were significant (P < 0.01), we noted that the VEGF levels in NeuYD × VEGF tumors remained well above (~10-fold increase) that found in tumors from untreated NeuYD mice (Fig. 2A). Collectively, these results suggest that the reduced expression of VEGF is not obligatorily linked to the antitumor activity of rapamycin.

To examine the effect of rapamycin on tumor vascularity, tissue sections were stained with α-CD31 antibody to illuminate vascular endothelial cells (Fig. 2B) and with α-NG2 antibody to detect vascular pericytes (Fig. 2C) in NeuYD × VEGF tumor tissue (24, 27). Pericytes are mesenchymal cells that make important contributions to the microvascular tree in both normal and pathologic tissues (28). Rapamycin treatment strongly suppressed both CD31

![Figure 2](https://cancerres.aacrjournals.org)
and NG2 staining in these samples. Rapamycin treatment reduced the average CD31 staining intensity by 35% in NeuYD tumors and by 46% in NeuYD × VEGF tumors. The apparent decrease in pericyte density in the rapamycin-treated tumor tissues was even more dramatic, with an average reduction of NG2 staining intensity of >7-fold.

In progressively growing tumors, hypoxic stress stimulates angiogenesis, in part, through the induction of the HIF-1 transcription factor (29). The rate-limiting step in the activation of HIF-1 is normally the accumulation of the HIF-1α subunit, which is mediated through hypoxia-induced stabilization of this labile protein. Immunohistochemical analyses of both NeuYD and NeuYD × VEGF tumor tissues revealed focal regions of intense HIF-1α staining, which presumably reflect localized zones of tissue hypoxia (Fig. 2D; data not shown). Treatment with rapamycin dramatically reduced the levels of HIF-1α in both NeuYD and NeuYD × VEGF tumors. These results suggest that inhibition of HIF-1-dependent gene expression is an important contributing factor to the anticancer activity of rapamycin.

**Antiproliferative and proapoptotic activity of rapamycin.** Studies in cultured cell lines have shown that rapamycin is a potent inhibitor of G1-S-phase progression in cycling cell populations (3, 7). To examine more directly the effect of rapamycin on mammary tumor cell proliferation in vivo, we treated tumor-bearing NeuYD × VEGF mice for 5 days with rapamycin and injected the animals with BrdUrd to mark cells that were actively replicating DNA. This short course of rapamycin therapy caused a partial but significant (P < 0.05) reduction in the proportion of BrdUrd-positive nuclei in established mammary tumors, indicating that this drug suppresses malignant cell proliferation in vivo (Fig. 3A).

In addition to its cytostatic effects, rapamycin induces apoptosis in certain cell types under specific culture conditions (3). To determine whether rapamycin therapy triggered apoptosis of ErbB2-driven cancer cells in vivo, we examined NeuYD tumor...
slices for DNA fragmentation by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining. Tumor tissue from animals treated with the drug vehicle displayed scattered TUNEL-positive foci (Fig. 3B). In contrast, samples from rapamycin-treated NeuYD mice exhibited islands of apoptotic cells. These results indicate that rapamycin inhibits in vivo tumor mass accumulation through both cytostatic and cytolytic mechanisms.

**Effects of rapamycin on protein phosphorylation and expression.** To further define the antitumor mechanism of rapamycin, we did a series of immunoblotting experiments with tumor-derived extracts from NeuYD × VEGF mice exposed to a short course of rapamycin therapy (0.75 mg/kg/d for 5 days). As expected, the phosphorylation of 4E-BP1, a known substrate for mTOR (30), was strongly inhibited by rapamycin (Fig. 4A and B). Similarly, the increased expression of the cyclin-dependent kinase inhibitor, p27Kip1, and the reduced level of cyclin D1 protein observed in rapamycin-treated tumor extracts were consistent with previous results obtained with cultured cell lines (31, 32). However, two unexpected outcomes of rapamycin treatment were the reductions in AKT phosphorylation and ErbB3 protein expression. The phospho-AKT antibodies react with the phosphorylated Ser173 site and mark the catalytically activated form of this protein kinase. The suppression of AKT activation may be linked to reduced ErbB3 expression, as ligand-induced heterodimerization of ErbB2 (NeuYD in the present context) with ErbB3 generates a powerful transmembrane signal for PI3K activation (16, 18). Thus, the anticaner activity of rapamycin against ErbB2-driven tumors may be due to both the direct inhibition of mTOR and the indirect down-regulation of ErbB3-dependent PI3K activation.

**Antiproliferative effects of rapamycin in monolayer versus spheroid cultures.** The in vivo antitumor activity of rapamycin likely reflects direct actions of the drug on the malignant cells as well as indirect effects on the tumor microenvironment. To focus on the tumor cell autonomous effects of rapamycin, we derived a series of epithelial cell lines from NeuYD and NeuYD × VEGF tumor tissues. Two of the cell lines from each tumor type were selected for further analysis. In the initial studies, we examined the antiproliferative effects of rapamycin under standard monolayer culture conditions. Considering its striking effects on tumor growth in vivo, rapamycin caused a surprisingly

![Figure 4. Protein expression in NeuYD × VEGF tumors. A. NeuYD × VEGF mice were given a short course of rapamycin therapy as described in Fig. 3 legend. Tumor extracts were immunoblotted with the indicated antibodies. B. summary of protein expression results. Photographic films from protein immunoblots were subjected to quantitative densitometric analysis with NIH Image 1.63 software. The densitometric units obtained for each protein were normalized to those obtained with the tubulin control in each sample. The normalized densitometric units were then arbitrarily set to 100% for the control vehicle-treated samples. Columns, mean from five independent tumor specimens in each treatment group; bars, SE.](image-url)
modest reduction (~30% decrease) in cell proliferation in monolayer cultures (Fig. 5A). However, recent studies have highlighted three-dimensional (spheroid) culture systems as more faithful models of tumor growth in vivo (33). Consequently, we reexamined the effects of rapamycin on the NeuYD-derived and NeuYD × VEGF-derived cell lines under three-dimensional culture conditions. In these experiments, the effect of rapamycin on cell growth was determined by measurement of spheroid volumes. In contrast to the results obtained with monolayer cells, rapamycin dramatically suppressed the growth of NeuYD and NeuYD × VEGF spheroids in Matrigel (Fig. 5B). Thus, relative to monolayer cultures, the three-dimensional spheroids displayed a marked increase in sensitivity to the antiproliferative and/or cytotoxic effects of rapamycin.

In subsequent studies, we compared the phosphorylation states and expression of several proteins related to mTOR signaling in
monolayer versus three-dimensional cultures (Fig. 5C). The phosphorylation of S6K1 kinase was effectively inhibited by rapamycin under both culture conditions, indicating that the inhibition of S6K1 kinase activity is not correlated with the magnitude of the antiproliferative effect of rapamycin. In contrast, rapamycin treatment dramatically decreased the phosphorylation of 4E-BP1 at Thr\(^{70}\) in three-dimensional spheroids but not in the same cells cultured under monolayer conditions. The reduced phosphorylation of 4E-BP1 was evidenced by both the decrease in α-phospho-Thr\(^{70}\) immunoreactivity and the disappearance of the more slowly migrating protein bands, which represent more highly phosphorylated forms of 4E-BP1 (34). The dephosphorylated form of 4E-BP1 binds to the initiation factor, eIF-4E, and thereby inhibits cap-dependent translation in mammalian cells (35). These results suggest that the suppression of eIF-4E-dependent translation may be causally related to the potent growth-inhibitory effects of rapamycin on YD A15 and YD A19 spheroids. Moreover, monolayer cultures of the same cells may be more resistant to the growth-inhibitory effect of rapamycin because they are better able to maintain 4E-BP1 phosphorylation, and in turn normal levels of eIF-4E-dependent protein synthesis, in the presence of this drug.

**Rapamycin induces apoptotic cell death.** As shown in Fig. 3C, rapamycin treatment led to the appearance of TUNEL-positive regions in tumors from NeuYD mice. To determine whether rapamycin induced apoptotic cell death in vitro, we measured the appearance of early-stage apoptotic (Annexin V–positive, propidium iodide–negative) cells by flow cytometry. Rapamycin treatment had little effect on the numbers of apoptotic cells in monolayer cultures. In contrast, cells isolated from multicellular spheroids displayed a clear peak of Annexin V–positive cells in the absence of drug, and the number of these cells was substantially increased (35% over control) by rapamycin exposure. We were not able to assay late apoptotic (Annexin V–positive, propidium iodide–positive) cells due to the lysis of these cells during the preparation of single-cell suspensions from the drug-treated spheroids.

To further document the proapoptotic activity of rapamycin in three-dimensional cell cultures, we examined cell extracts for evidence of poly(ADP-ribose) polymerase (PARP) cleavage (Fig. 6B). Full-length PARP (molecular mass, 115 kDa) is cleaved by activated caspases to yield a primary cleavage product bearing a molecular mass of 85 kDa. Rapamycin exposure triggered no detectable PARP cleavage in monolayer cell cultures, whereas cells residing in spheroids displayed reduced expression of full-length PARP and the concomitant appearance of a faint band corresponding to the initial caspase-mediated PARP cleavage product. Further evidence of apoptosis in rapamycin-treated spheroids came from immunoblotting experiments showing loss of the caspase-3 proenzyme, a marker for caspase-3 activation, and a concomitant increase in caspase-3 activity in cell extracts (Fig. 6B). We conclude from the results in Figs. 5 and 6 that rapamycin suppresses the growth of NeuYD-expressing spheroids through a combination of reduced cell proliferation and increased cell death.

**Effect of rapamycin on ErbB3 expression.** The finding that rapamycin therapy decreased ErbB3 expression in NeuYD-positive mammary tumors in vivo prompted an investigation of ErbB3 protein levels in control and drug-treated YD A15 and YD A19 cell spheroids (Fig. 7A). Both cell lines expressed the ErbB3 polypeptide, and rapamycin treatment clearly decreased ErbB3 but not ErbB2 expression in these cells. These results were not unique to the murine mammary epithelial cell lines, as rapamycin exposure also reduced the amount of ErbB3 protein expressed in the human breast carcinoma cell line MDA-MB-231. To determine whether
rapamycin affected ErbB3 gene expression at the mRNA level, we did Q-PCR analysis with total cellular RNA prepared from YD A15 and YD A19 cells grown under three-dimensional culture conditions. The Q-PCR data showed that rapamycin had no effect on the steady-state level of ErbB3 mRNA in these cells (paired t test, $P > 0.17$), indicating that this drug decreases ErbB3 protein expression through a post-transcriptional mechanism. Technical limitations related to the available antibody reagents prevented examination of ErbB3 mRNA translation by pulse labeling with $^{35}$S-labeled amino acids. Given that rapamycin is a known inhibitor of elf-4E-dependent mRNA translation, we consider translational suppression to be the most likely mechanism underlying the reduction in ErbB3 protein levels in the drug-treated YD A15 and YD A19 cell lines.

**Effects of rapamycin on hypoxia-induced hypoxia-inducible factor-1 activation.** Rapamycin therapy significantly decreased the expression of HIF-1α in NeuYD and NeuYD/C2VEGF tumor tissues (Fig. 2E) and provoked a corresponding decrease in the intratumoral expression of the GLUT1 glucose transporter (Fig. 8A), which is transcriptionally regulated by HIF-1 (36). In subsequent in vitro experiments, we cultured YD A15 and YD A19 cells under normoxic (20% $O_2$) or hypoxic (1% $O_2$) conditions and immunoblotted cell extracts with HIF-1α- or GLUT1-specific antibodies. In normal cells, HIF-1α protein expression is maintained at low levels in normoxic conditions due to continuous degradation via the ubiquitin-proteasome pathway (36). However, both of the NeuYD tumor-derived clones tested in this study showed relatively high levels of HIF-1α expression under normoxic conditions, and exposure to hypoxia caused only a marginal increase over the basal HIF-1α expression level in these cells (Fig. 8A). Treatment of normoxic or hypoxic cells with rapamycin reduced both HIF-1α expression and HIF-1-dependent transcriptional activity by ~50% (Fig. 8B and C). We noted that, in spite of the presence of substantial HIF-1α protein in normoxic YD A15 and YD A19 cells, transcription of the HIF-1-dependent reporter gene remained strongly dependent on reduced oxygen tension (see Discussion). Consistent with the HIF-1 reporter gene assays, treatment of the hypoxic YD A15 and YD A19 cells with rapamycin substantially reduced endogenous GLUT1 expression in these cells (Fig. 8A). These results substantiate previous reports, which showed that rapamycin effectively inhibits HIF-1-mediated gene expression in human cancer cell lines (25, 37).

**Discussion**

The present findings show that rapamycin is a potent inhibitor of tumor growth in two related mouse models of ErbB2-positive human breast cancer. Previous results in mice bearing orthotopically implanted colon carcinoma cells suggested that suppression of angiogenesis played a major role in the in vivo anticancer activity of rapamycin and that the antiangiogenic activity of this drug was explained, at least in part, by the inhibition of VEGF production by the malignant cells (21). Our results with NeuYD×VEGF mice show that rapamycin retains significant antitumor activity in the setting of supraphysiologic levels of VEGF, indicating that high-level VEGF production was not sufficient to render tumor growth resistant to rapamycin. The disruption of tumor neovascularization observed in rapamycin-treated NeuYD×VEGF mice suggests that, in addition to its tumor cell autonomous activities, rapamycin exerts therapeutically relevant actions on the tumor microenvironment, specifically on host endothelial cells and pericytes.

Treatment of NeuYD and NeuYD×VEGF mice with rapamycin before the emergence of palpable mammary tumors dramatically reduced both the overall numbers of tumors and the size of those...
tumors that did emerge in the drug-treated animals. These chemopreventive effects of rapamycin were observed at drug doses that caused no detectable adverse effects in the mice. From a clinical perspective, a chronic reduction in mTOR activity could be beneficial for certain high-risk cancer patients, particularly as adjuvant therapy for the suppression of tumor reoccurrence after successful removal of primary tumors. Current evidence suggests that the best candidates for such therapy would be patients whose primary tumors bear abnormalities leading to hyper-activation of the PI3K signaling pathway (38). Prophylactic therapy with a mTOR inhibitor may also be a promising strategy for the prevention of disease relapse in patients with ErbB2-positive breast cancer.

Initiation of rapamycin therapy after the emergence of palpable (\( \sim 5 \) mm) tumors indicated that mTOR inhibition strongly decreased the rate of tumor growth. However, we noted that tumor growth resumed shortly after cessation of drug therapy, indicating that the overall effect of rapamycin is tumoristatic rather than curative, at least when given as a single agent. Low-dose therapy with rapamycin or related compounds may have a risk-benefit profile that would make these drugs suitable candidates for long-term administration in certain patients, consistent with the idea that some cancers should be treated as chronic diseases (39). Alternatively, if curative therapy is the objective, then the present findings suggest that mTOR inhibitors will be used most effectively in combination with more conventional cytoreductive therapies, including surgery, cytotoxic chemotherapy, and radiation.

Our results underscore the importance of the tumor microenvironment as a target for the anticancer activity of rapamycin. Administration of rapamycin significantly reduced the numbers of CD31-positive endothelial cells and NG2-positive pericytes in mammary tumor tissues from NeuYD and NeuYD \( \times \) VEGF mice. The effects of rapamycin on pericycle migration and/or proliferation into the tumor tissue were particularly striking. Pericytes are microvasculature-associated mesenchymal cells that are believed to fulfill the structural and contractile functions done by vascular smooth muscle cells in larger vessels (28). The interaction between endothelial cells and pericytes is critical for the formation of a structurally sound microvasculature. The profound inhibitory effect of rapamycin on pericycle density in tumor tissues may explain the previously reported antiangiogenic action of this drug in mice bearing orthotopic tumor implants (21). This model is also consistent with the striking clinical benefits observed with rapamycin-coated stents, which block restenosis through selective inhibition of vascular smooth muscle cells rather than endothelial cells (40). Nonetheless, rapamycin is known to disrupt signaling through VEGF and other cytokine receptors (41), and direct effects of the drug on the proliferation and/or migration of CD31-positive endothelial cells cannot be ruled out based on the available results.

Previous results from this laboratory and others indicated that rapamycin inhibited both the expression of HIF-1\( \alpha \) and the HIF-1-mediated transcriptional activity in various cancer cell lines cultured as cell monolayers under hypoxic conditions (25, 37). The gene expression program controlled by HIF-1 is critical for cellular adaptation to hypoxic stress and promotes accelerated growth of solid tumors. Consequently, HIF-1 is considered a potential target for the development of novel anticancer agents (36). The present findings extend the earlier in vitro evidence for the HIF-1-inhibitory effects of rapamycin and suggest that the in vivo antitumor activities of rapamycin and related mTOR inhibitors are attributable, in part, to interference with HIF-1 activation and function in solid tumors. Immunohistochemical analyses of mammary tumor tissues from NeuYD mice as well as

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5 Unpublished observations.
NeuYD × VEGF mice (data not shown) revealed focal regions of intense staining for the HIF-1α protein, which presumably correspond to islands of hypoxic tumor cells. Rapamycin treatment strongly reduced expression of both the HIF-1α subunit and the HIF-1 target gene, GLUT1, in NeuYD tumor tissue. Notably, high level expression of HIF-1α and GLUT1 in human tumors is considered a poor prognostic indicator for therapeutic responsiveness and patient survival (42, 43). Although a causal relationship between the HIF-1-inhibitory and the antitumor activities of rapamycin has not been proven, the present findings suggest that hypoxic tumor tissues may be particularly sensitive to rapamycin or other mTOR inhibitors.

Studies done in transformed epithelial cell lines derived from NeuYD tumor tissue revealed that rapamycin also interferes with HIF-1 function by inhibiting the transactivating function of the HIF-1α-HIF-1β heterodimer. Examination of HIF-1α protein expression in the NeuYD A15 and A19 cell lines revealed that these cells contained relatively high levels of this protein under normoxic culture conditions. Like the transgene-expressing tumor tissue from which they were derived, these cells express an activated ErbB2 receptor mutant, a phenotype that has been linked previously to constitutively elevated levels of HIF-1α (37). However, reporter gene assays showed that the transactivating function of HIF-1 remained strongly dependent on hypoxia in spite of the elevated basal level of HIF-1α. This result is consistent with the existence of an additional, oxygen-dependent regulatory mechanism that impinges on the carboxyl-terminal transactivation domain (CTAD) of HIF-1α (44). Under normoxic conditions, the CTAD is modified by an asparagine N-hydroxylase, thereby blocking the interaction between HIF-1α and transcriptional coactivating proteins. The activity of this enzyme is inhibited by hypoxia, allowing the accumulation of transcriptionally competent HIF-1. In hypoxic NeuYD cells, we found that suppression of HIF-1-dependent transcription was significantly inhibited by rapamycin. An open question that remains to be addressed is whether rapamycin causes sustained modification of the HIF-1α CTAD or alters another component of the HIF-1-associated transcriptional machinery in hypoxic tumor cells.

An unexpected outcome of these experiments was that treatment with rapamycin suppressed AKT activation in tumor tissues from NeuYD × VEGF mice. A plausible mechanism for this effect emerged with the discovery that expression of the EGF receptor family member, ErbB3, was reduced by ~50% in the same samples. A notable feature of the ErbB3 cytoplasmic domain is the presence of seven tyrosine-containing motifs that represent potential binding sites for the p85 subunit of PI3K (45). Because the ErbB3 subunit lacks intrinsic tyrosine kinase activity, phosphorylation of these cytoplasmic tyrosine residues requires heterodimerization of ErbB3 with ErbB2 or other ErB family members. In the NeuYD transgenic mice, suppression of ErbB3 expression by rapamycin might decrease the coupling efficiency between the activated ErbB2 receptors and the PI3K signaling cascade in malignant epithelial cells. Consequently, ErbB3 could represent an indirect but critically important target of mTOR inhibitors in ErbB2-positive breast cancers.

Our in vitro studies with NeuYD spheroids showed that rapamycin strongly suppressed ErbB3 protein expression but had no effect on the steady-state level of ErbB3 mRNA. Although we were unable to measure directly the effect of rapamycin on ErbB3 mRNA translation, the most likely explanation for these findings is that ErbB3 protein synthesis is particularly sensitive to inhibition of mTOR in these cells. The observed decreases in ErbB3 expression were not unique to the murine NeuYD spheroids, as ErbB3 protein levels were also reduced by exposure of human MD-MB-231 breast carcinoma cells to rapamycin. Certain mRNAs are under tight translational control by the mTOR signaling pathway due to a strong dependence on the activity of the cap-binding protein, eIF-4E (35). Our results suggest that ErbB3 should be added to the list of growth-regulatory proteins (e.g., cyclin D1 and c-Myc) that are translationally regulated by the mTOR signaling pathway.

An unresolved issue is whether drug-induced cell death plays a major role in the in vivo antitumor activity of rapamycin. We observed localized clusters of TUNEL-positive cells in rapamycin-treated tumor tissues, indicating that a subpopulation of the tumor cells had undergone apoptosis. However, in the in vivo setting, it is not possible to differentiate between tumor cell autonomous effects of the drug on cell viability and tumor cell extrinsic actions on the tumor microenvironment, leading to a bystander apoptotic response in the malignant cells. Previous studies showed that rapamycin triggers a p53-independent apoptotic response in certain cell types under specified culture conditions (3). Our in vitro experiments with monolayer cultures of NeuYD tumor-derived cell lines documented a modest inhibitory effect of rapamycin on cell proliferation with no evidence of cell death. However, three-dimensional cultures of the same cell lines displayed clear biochemical evidence of apoptotic cell death after exposure to rapamycin. These results support the idea that rapamycin triggers a tumor cell autonomous apoptotic response during in vivo therapy and that both cell death and cytostasis contribute to the inhibition of tumor growth observed in drug-treated mice. Nonetheless, we cannot rule out other mechanisms of cell death in rapamycin-treated tumors, including “programmed necrosis” or autophagic death (46). Unlike apoptosis, these two death processes are tightly linked to bioenergetic stress. Inhibition of mTOR has been associated with both a decrease in glucose consumption (a major source of metabolic energy) and an increased autophagic activity (8, 47). Thus, additional experiments will be needed to determine the relative roles of apoptosis, necrosis, and autophagy in the antitumor activity of rapamycin.

In summary, the present findings suggest that the antitumor activity of rapamycin stems from a complex array of effects on both the tumor cells themselves and the host tissues that support tumor growth. These results support the idea that rapamycin could have important therapeutic applications in patients with ErbB2-positive breast cancers. Finally, our findings add to the growing body of evidence that three-dimensional cultures of human cancer cells are superior in vitro model systems for studies of tumor cell biology and mechanisms of anticancer drug action (33).

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References

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