Targeting Mammalian Target of Rapamycin Synergistically Enhances Chemotherapy-Induced Cytotoxicity in Breast Cancer Cells

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ABSTRACT

Purpose: The serine-threonine kinase mammalian target of rapamycin has emerged as a potential target for cancer therapy. Rapamycin and rapamycin analogs are undergoing clinical trials and have induced clinical responses in a subgroup of patients. Rapamycin has also been reported to enhance the efficacy of several cytotoxic agents. The aim of this study was to determine the nature of the interactions between rapamycin and chemotherapeutic agents used as first- and second-line agents against breast cancer.

Experimental Design: We performed a multiple drug effect/combination index isobologram analysis in cells sensitive and resistant to rapamycin alone in vitro, and we evaluated the in vivo efficacy of combination therapy in a rapamycin-sensitive model.

Results: In vitro, synergistic interactions were observed in combinations with paclitaxel, carboplatin, and vinorelbine. Additive effects were observed in combinations with doxorubicin and gemcitabine. Rapamycin dramatically enhanced paclitaxel- and carboplatin-induced apoptosis. This effect was sequence dependent and mediated at least partly through caspase activation. Furthermore, rapamycin-enhanced chemosensitivity to paclitaxel and carboplatin in HER2/neu-overexpressing cells, suggesting a potential approach to these poorly behaving tumors. Cell lines that are resistant to the growth-inhibitory effect of rapamycin were also resistant to rapamycin-mediated chemosensitization. In vivo, rapamycin combined with paclitaxel resulted in a significant reduction in tumor volume compared with either agent alone in rapamycin-sensitive tumors.

Conclusions: Rapamycin potentiates the cytotoxicity of selected chemotherapeutic agents in cell lines sensitive to the effects of rapamycin due to aberrations in the phosphatidylinositol 3′-kinase/Akt pathway, suggesting that combination therapy may be effective in patients selected for aberrations in this pathway.

INTRODUCTION

Rapamycin, a lipophilic macrolide antibiotic, was originally isolated from the soil bacterium Streptomyces hygroscopicus in the mid-1970s (1). Although it was initially developed as a fungicide and immunosuppressant, investigators found that it had potent antitumor activity in a variety of solid tumors (2). Rapamycin inhibits the serine-threonine kinase mammalian target of rapamycin (mTOR) by binding to the immunophilin FK506-binding protein 12. The inhibition of mTOR kinase leads to dephosphorylation of its two major downstream signaling components, p70 S6 kinase (S6K1) and 4E-BP1 (3). In turn, dephosphorylation of S6K1 and 4E-BP1 inhibits the translation of specific mRNAs involved in cell cycle and proliferation and leads to G1 growth arrest.

Mammalian target of rapamycin appears to be downstream of phosphatidylinositol 3′-kinase (PI3K)/Akt in a linear pathway, although Akt also regulates mTOR through the tuberous sclerosis gene products TSC1 and TSC2, which inhibit mTOR and are also targets for Akt (4–7). The PTEN (phosphatase and tensin homologue deleted from chromosome 10) tumor suppressor gene is a phosphatase that negatively regulates PI3K signaling. PTEN is mutated or decreased in several types of sporadic tumors, including breast cancers, and in patients with Cowden’s breast cancer predisposition syndrome. Furthermore, the PI3K/Akt pathway is activated in breast cancer cells via HER2/neu overexpression, as well as activation of insulin-like growth factor and integrins (8–10). In our previous work, we have shown that breast cancer cells with phosphorylated Akt consistent with activation of the PI3K/Akt pathway, as well as cells with overexpression of S6K1, are sensitive to rapamycin (11). Therefore, the mTOR signaling pathway is likely to be aberrantly activated in a substantial number of breast tumors, making mTOR an especially promising target for breast cancer therapy.

The antitumor activity of rapamycin as a single agent has been described in vitro and in vivo. Most reports have shown that rapamycin acts as a cytostatic agent by arresting cells in the
G1 phase, although in certain experimental systems, rapamycin alone induces apoptosis in cells lacking functional p53 (12, 13). When combined with other chemotherapeutic agents, rapamycin and rapamycin analogs have been reported to increase the efficacy of a variety of cytotoxic agents, including cisplatin, doxorubicin, camptothecin, 5-fluorouracil, cyclophosphamide, and 1-β-D-arabinofuranosylcytosine, in several cancer cell types (14–17). However, the nature of the interactions between rapamycin and these chemotherapeutic agents has not been systematically studied in rapamycin-sensitive and -resistant breast cancers.

The objective of our study was to determine whether rapamycin enhances the efficacy of chemotherapeutic agents commonly used in breast cancer and determine the nature of the interactions between rapamycin and other chemotherapeutic agents. These results will provide guidance regarding how to best combine rapamycin with existing cancer therapeutic agents. Here, we report that in vitro rapamycin has a synergistic effect with paclitaxel, carboplatin, and vinorelbine and an additive effect with doxorubicin and gemcitabine. We show that rapamycin enhances chemotherapy-induced apoptosis at least partly through caspase activation. Furthermore, we show that rapamycin in combination with paclitaxel leads to a significant reduction in tumor growth in vivo. Together, these results suggest that rapamycin in combination with selected cytotoxic agents may provide an effective alternative for breast cancer therapy in a selected population of patients.

MATERIALS AND METHODS

Cell Lines and Cultures. MCF-7, MDA-MB-468, MDA-MB-231, NCI/ADR-RES, and NIH3T3 cell lines were obtained from American Type Culture Collection (Manassas, VA). The NIH3T3/HER2/neu cell line (referred to henceforth as NIH3T3/HER2) has been described previously (8). The cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, and 1% penicillin-streptomycin at 37°C and humidified in 5% CO2.

Cell Treatment and Cell Proliferation Assays. To test the effect of rapamycin in combination with other chemotherapeutic agents on cell proliferation, cells were plated into 96-well, flat-bottomed plates at 2 to 4 × 103 cells per 100 μL per well, with density determined on the basis of the growth characteristics of each cell line. After overnight incubation, triplicate wells were treated with varying concentrations of rapamycin ranging from 0.1 to 100 nmol/L for 4 days. The concentrations of the chemotherapeutic agents varied from 0.01 to 10 μg/mL. The relative percentages of metabolically active cells compared with untreated controls were then determined on the basis of the mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazone. Results were assessed in a 96-well format plate reader by measuring the absorbance at a wavelength of 540 nmol/L (A540 nm).

Dose-Effect Analyses. Rapamycin was combined with each of the different chemotherapy agents at a fixed ratio that spanned the individual IC50 of each drug. The IC50 was determined on the basis of the dose-response curves using a standard MTT assay. Median-effect plot analyses and calculation of the combination index (CI) were analyzed by the method of Chou and Talalay (18). The commercial software package was obtained from Calcusyn (Biosoft, Cambridge, United Kingdom).

Clonogenic Assay. Cells (2,000–4,000 cells per well) were seeded on 6-well plates. After cells had been incubated for 24 hours, rapamycin, paclitaxel, or both were added. The culture medium was changed every 2 days with 10% fetal bovine serum until we stained the colonies with crystal violet on day 16.

Annexin V Binding. Apoptosis was determined using the ApoAlert annexin V apoptosis kit (Clontech, Palo Alto, CA) according to the manufacturer’s protocol. In brief, cells were trypsinized and rinsed in binding buffer. They were then resuspended in 200 μL of binding buffer, to which 5 μL of annexin V (20 μg/mL in Tris-NaCl buffer) and 10 μL of propidium iodide (1 μg/mL) were added. Cells were incubated for 10 to 15 minutes at ambient temperature in the dark and then analyzed by flow cytometry.
**Western Blot Analysis.** Cultured cells were washed with cold PBS and lysed in lysis buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 0.5% Nonidet P-40, 50 mmol/L NaF, 1 mmol/L Na<sub>2</sub>VO<sub>4</sub>, and 25 μg/mL aprotinin. Cell lysates containing 40 μg of protein were separated by 8% SDS-PAGE. Immunoblots were visualized using the enhanced chemiluminescence detection kit (ECL; Amersham, Arlington Heights, IL). Antibodies against poly(ADP-ribose) polymerase (PARP), phospho-Akt (Ser<sup>473</sup>), total Akt, phospho-S6K1 (Thr<sup>389</sup>), total S6K1, phospho-S6 protein, total S6 protein, phospho-4E-BP1 (Ser<sup>65</sup>), and total 4E-BP1 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). β-Actin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO).

**In vivo Studies.** All animal studies were conducted according to the guidelines of the American Association of Laboratory Animal Care. Four- to six-week-old female nude mice (Harlan Sprague Dawley) were subcutaneously inoculated with MDA-MB-468 cells in the mammary fat pad. In the first experiment, each group consisted of five mice. The first group received vehicle (PBS) alone. The second through sixth groups were given intraperitoneal paclitaxel (5 mg/kg), rapamycin (15 mg/kg), paclitaxel (5 mg/kg) + rapamycin (15 mg/kg), and paclitaxel (5 mg/kg) + rapamycin (15 mg/kg). In the second experiment, each group consisted of six mice. The first group received PBS only. The other groups were given intraperitoneal paclitaxel (5 mg/kg) alone, rapamycin (15 mg/kg) alone, rapamycin (15 mg/kg) and paclitaxel (5 mg/kg) synchronously, or paclitaxel (5 mg/kg) followed by rapamycin (15 mg/kg) 24 hours later. Treatments were given once a week for 6 weeks. Tumor volumes were calculated using the formula

\[ V = \pi a^2 b \]

where \( a \) was the short axis, and \( b \) was the long axis.

**RESULTS**

**Rapamycin Is Synergistic with Paclitaxel, Carboplatin, and Vinorelbine.** We sought to determine the effect of rapamycin in combination with five first- and second-line chemotherapeutic agents commonly used to treat breast cancer. These five agents have different mechanisms of action and cell phase specificities [paclitaxel (a taxane, M phase), carboplatin (a DNA-binding alkylating agent; nonspecific cell cycle phase), vinorelbine (a Vinca alkaloid; M phase), doxorubicin (anthracycline; cell cycle phase nonspecific), and gemcitabine (a pyrimidine analog; S-phase; ref. 19)]. In most studies, rapamycin has been demonstrated to induce a G<sub>1</sub> arrest (11); however, it can induce apoptosis in a subset of tumor lineages (12, 13, 17) at least partly dependent on p53 status, suggesting the potential for additive, synergistic, or inhibitory effects when combined with these drugs.

The rapamycin-sensitive (11) cell lines MCF-7 (p53 wild-type, PTEN wild-type) and MDA-MB-468 (p53 mutant, PTEN mutant) were treated with serial dilutions of rapamycin and the various chemotherapeutic agents at fixed ratios spanning the IC<sub>50</sub> of each drug. After 4 days of exposure, growth inhibition was measured with a MTT assay. In this assay, CI values are calculated for different dose-effect levels on the basis of parameters derived from median-effect plots of rapamycin alone, the chemotherapeutic agent alone, and the combination of the two at fixed molar ratios. A CI value significantly less than 1 indicates synergy, a CI not significantly different from 1 indicates addition, and a CI significantly higher than 1 indicates antagonism (18). Synergy is defined as a combination of two drugs that has a greater therapeutic effect than what would be expected by the simple addition of the effects of each drug. The CI values at the IC<sub>50</sub> for MCF-7 cells were 0.375 for paclitaxel (\( P = 0.001 \)), 0.582 for carboplatin (\( P = 0.003 \)), 0.686 for vinorelbine (\( P = 0.003 \)), 0.922 for doxorubicin (\( P = 0.006 \)), and 1.25 for gemcitabine (\( P = 0.025 \); Fig. 1). The CI values at the IC<sub>50</sub> for MDA-MB-468 cells were 0.350 for paclitaxel (\( P = 0.003 \)), 0.522 for carboplatin (\( P = 0.004 \)), 0.706 for vinorelbine (\( P = 0.04 \)), 1.24 for doxorubicin (\( P = 0.003 \)), and 1.40 for gemcitabine (\( P = 0.004 \)).

![Fig. 2](https://clinicalcancerres.aacrjournals.org/del.png)

**Fig. 2** Paclitaxel enhances rapamycin-mediated cell growth inhibition. A, MCF-7 cells were treated with rapamycin and paclitaxel at varying concentrations for 4 days. Cell growth inhibition was determined by MTT assay. B, MDA-MB-468 cells were treated with rapamycin and paclitaxel at varying concentrations for 4 days. Dose-dependent cell growth inhibition was determined by a MTT assay. Paclitaxel concentrations in A and B are as follows: □ 0 μg/mL; □ 0.01 μg/mL; □ 0.1 μg/mL; □ 1 μg/mL; □ 10 μg/mL. C: A clonogenic assay was performed to analyze anchorage-dependent growth. Surviving colonies were counted 16 days after treatment. The growth inhibition induced by paclitaxel alone and 100 nmol/L rapamycin alone (1) was compared with the combination of paclitaxel + 10 nmol/L rapamycin (2). The growth inhibition induced by paclitaxel alone and 100 nmol/L rapamycin alone (3) was compared with the combination of paclitaxel + 100 nmol/L rapamycin (4). The results shown are the mean of three independent experiments. □, 0.1 μg/mL Paclitaxel; □, 10 nmol/L rapamycin; ◻, 0.1 μg/mL Paclitaxel + 100 nmol/L rapamycin; □, 100 nmol/L rapamycin; □, 0.1 μg/mL Paclitaxel + 100 nmol/L rapamycin.
for doxorubicin ($P = 0.13$), and 0.962 for gemcitabine ($P = 0.28$). Thus, when rapamycin was combined with paclitaxel, vinorelbine, or carboplatin, synergistic effects were found in both rapamycin-sensitive cell lines. When rapamycin was combined with doxorubicin and gemcitabine, the effects were additive.

**Rapamycin Enhances the Growth-Inhibitory Effects of Paclitaxel.** To examine the sensitivity of MCF-7 and MDA-MB-468 cells to the combination of rapamycin and paclitaxel, cells were treated with varying concentrations of each agent for 4 days. Cell proliferation was measured by MTT assay, and the percentage of growth inhibition was calculated and standardized to that of untreated controls. The combination of rapamycin and paclitaxel exhibited greater dose-dependent inhibition of cell proliferation than did each agent alone in both cell lines (Fig. 2A and B).

The effect of rapamycin in combination with paclitaxel on anchorage-dependent growth was further evaluated with a clonogenic assay. MDA-MB-468 cells were treated with paclitaxel (0.1 µg/mL) alone and in combination with rapamycin (10 and 100 nmol/L), and colonies were counted after 16 days of treatment. The combination of rapamycin and paclitaxel inhibited anchorage-dependent growth more than the additive effects of either agent alone (Fig. 2C).

**Rapamycin Increases Paclitaxel and Carboplatin-Induced Apoptosis.** We next examined whether the synergistic interactions of rapamycin with paclitaxel and carboplatin were due to an increase in cytotoxic agent-induced apoptosis. One of the early events in apoptosis, the translocation of phosphatidylserine from the inner layer of the plasma membrane to the outer layer, can be detected by the binding of annexin V to

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**Fig. 3** Rapamycin enhances paclitaxel- and carboplatin-induced apoptosis. A. MDA-MB-468 cells were treated with 10 or 100 nmol/L rapamycin alone (top middle and top right panels), 0.01 µg/mL carboplatin alone (bottom left panel), or with both agents (bottom middle and right panels). Cells were harvested after 48 hours to determine apoptosis and examined by FACS analysis for annexin V-fluorescein isothiocyanate and propidium iodide fluorescence. The percentage of annexin-positive cells is shown in the top right corner of each panel. B, MCF-7 and MDA-MB-468 cells were treated with carboplatin (0.01 µg/mL), rapamycin, or both agents. Apoptosis was determined by annexin labeling. C, MCF-7 and MDA-MB-468 cells were treated with paclitaxel (0.1 µg/mL), rapamycin, or both agents. Apoptosis was determined by annexin labeling. Results shown are the mean of three independent experiments. C, carboplatin; P, paclitaxel; R, rapamycin.
the cell surface. Thus we evaluated the level of annexin labeling to MCF-7 and MDA-MB-468 cells after treatment with each agent alone and in combination. MDA-MB-468 cells were treated with carboplatin alone (0.01 μg/mL), rapamycin alone (10 or 100 nmol/L), or a combination of these agents (Fig. 3A). After 48 hours, the percentage of annexin-positive cells was determined by fluorescence-activated cell-sorting (FACS) analysis.

At the concentrations of carboplatin and rapamycin used, either agent alone did not induce a significant increase in apoptosis. Strikingly, the combination of rapamycin and carboplatin, at concentrations at which neither agent alone induced apoptosis, induced dramatic increases in the percentage of annexin-positive cells, indicative of induction of apoptosis. Similar results were obtained for MCF-7 and MDA-MB-468 cells in at least three independent experiments (Fig. 3B). Similar results were also obtained when rapamycin was combined with paclitaxel (0.1 μg/mL) under the same conditions (Fig. 3C). These results demonstrate that rapamycin enhances the efficacy of paclitaxel and carboplatin by increasing the degree of apoptosis induced by these chemotherapy agents.

**Rapamycin Enhances Apoptosis by Caspase Activation.** We hypothesized that the mechanism of rapamycin-enhanced, paclitaxel-induced apoptosis may involve caspase activation. To test this hypothesis, we treated MDA-MB-468 cells with a combination of rapamycin and paclitaxel in the absence and presence of the broad spectrum caspase inhibitor benzylxycarbonyl-VAD-fluoromethyl ketone (z-VAD-fmk). After 48 hours, the percentage of annexin-positive cells was reduced in the presence of z-VAD-fmk (Fig. 4A). These results suggest that enhancement by rapamycin of paclitaxel-induced apoptosis might be caspase dependent. To directly assess caspase activation, we evaluated cell death-related cleavage of the DNA repair enzyme PARP (20). Western blot analysis showed cleavage of the 116-kDa protein PARP to a 89-kDa fragment when rapamycin was combined with paclitaxel (Fig. 4B). The dimethyl sulfoxide (DMSO) vehicle did not induce apoptosis alone or when combined with paclitaxel. Taken together, these results suggest that activation of the caspase cascade contributes to the ability of rapamycin to enhance paclitaxel-induced apoptosis.

**Rapamycin Enhances Chemosensitivity in Rapamycin-Sensitive but not Rapamycin-Resistant Cell Lines.** We have demonstrated previously that the MCF-7 and MDA-MB-468 cell lines are sensitive to the growth-inhibitory effects of rapamycin (IC50 = 10 and 1 nmol/L, respectively; ref. 11). Therefore, we investigated whether the addition of paclitaxel to rapamycin would increase cytotoxicity in cells that are resistant to the growth-inhibitory effect of rapamycin as a single agent. We evaluated two cell lines, MDA-MB-231 and NCI/ADR-RES, which we previously showed to be relatively resistant to rapamycin (IC50 = 2,500 and 900 nmol/L, respectively; ref. 11).

Cells were treated with concentrations of paclitaxel (0.1 μg/mL) and rapamycin (100 nmol/L) that demonstrated synergy, both alone and in combination, in MCF-7 and MDA-MB-468 cells. After 48 hours, the percentage of annexin-positive cells was determined by FACS analysis. The data demonstrated that combined therapy with rapamycin and paclitaxel did not result in a higher degree of apoptosis in either of the rapamycin-resistant cell lines (Fig. 5).

To further clarify the interaction between rapamycin and paclitaxel in rapamycin-resistant cells, we determined the CI values. The CI values at the IC50 of rapamycin and paclitaxel were 1.17 for MDA-MB-231 cells (P = 0.22) and 1.18 for NCI/ADR-RES cells (P = 0.26). The effect of rapamycin was not synergistic with that of paclitaxel in either of these two rapamycin-resistant cell lines. These results suggest that cells that are resistant to the growth-inhibitory effect of rapamycin are also resistant to the ability of rapamycin to increase chemosensitivity.

**Rapamycin Enhances the Chemosensitivity of HER2/neu-Overexpressing Cells.** Overexpression of the receptor tyrosine kinase HER2/neu has been associated with paclitaxel and carboplatin resistance in breast cancer cells (21) as well as poor outcome in patients (22). We thus evaluated whether rapamycin could overcome chemoresistance conferred by HER2/neu. We first compared the sensitivity to paclitaxel and carboplatin of NIH3T3 cells with that of isogenic HER2/neu-transfected NIH3T3 cells (NIH3T3/HER2; Fig. 6). Consistent with our expectations, NIH3T3/HER2 cells were more resistant to paclitaxel and to carboplatin than were NIH3T3 cells (P < 0.0001). To determine the effect of rapamycin on paclitaxel-induced cell death, we treated NIH3T3/HER2 and parental NIH3T3 cells with increasing concentrations of paclitaxel (0.001 to 10 μg/mL) in the presence of 10 or 100 nmol/L rapamycin. After treatment for 4 days, cell viability was measured using a MTT assay. Although baseline measurements (without rapamycin) revealed statistically significant differences in inhibition between NIH3T3/HER2 and parental NIH3T3 cells, no statistically significant difference between the two cell
lines was found when they were treated with paclitaxel in the presence of either 10 or 100 nmol/L rapamycin \( (P = 0.66 \) and \( P = 0.13 \), respectively). Thus, concentrations of 10 and 100 nmol/L rapamycin overcame the chemoresistance conferred by NIH3T3/HER2 cells, resulting in an equivalent outcome in the normal and HER2-transfected cells. In the presence of 10 nmol/L rapamycin, NIH3T3 cells were more sensitive to carboplatin than were NIH3T3/HER2 cells, but when both cell lines were treated with carboplatin in the presence of 100 nmol/L rapamycin, there was no statistically significant difference in their chemosensitivity \( (P = 0.44)\).

We further evaluated the ability of rapamycin to overcome chemoresistance in NIH3T3/HER2 cells by examining the degree of apoptosis in both cell lines. Cells were treated with rapamycin alone (100 nmol/L), paclitaxel alone (1 or 10 \( \mu \)g/mL), or a combination of these agents. After 48 hours, the percentage of annexin-positive cells was determined by FACS analysis (Fig. 7). We found that rapamycin enhanced paclitaxel-induced apoptosis at both paclitaxel concentrations. However, even in the presence of rapamycin, the degree of apoptosis observed with paclitaxel treatment in the NIH3T3/HER2 cells was less than that observed in the NIH3T3 cells. Therefore, the data suggest that rapamycin only partially abrogates HER2/neu-induced chemoresistance, at least as assessed by apoptosis.

**Rapamycin in Combination with Paclitaxel Down-Regulates the mTOR Signaling Pathway.** To evaluate the effects of rapamycin combined with paclitaxel on the mTOR signaling pathway, we determined the expression and phosphorylation of targets of mTOR in MDA-MB-468 cells by Western blot analysis (Fig. 8). Rapamycin alone, as expected, decreased the phosphorylation of 4E-BP1 and S6K1 (major targets of mTOR) and the S6K1 phosphorylation target ribosomal S6 protein. Although paclitaxel has been previously shown to induce phosphorylation of S6K1 on threonine 421 and serine 424 (23), paclitaxel did not alter the phosphorylation of threonine 389 in MDA-MB-468 cells. Rapamycin in combination with paclitaxel down-regulated mTOR signaling, similar to rapamycin alone. Neither single agent alone nor the combination altered the Akt phosphorylation status. These
results suggest that dephosphorylation of key downstream molecules in the mTOR signaling pathway may potentiate the cytotoxic effect of paclitaxel.

**Rapamycin Enhances Paclitaxel Cytotoxicity in a Sequence-Dependent Manner.** The synergistic interactions of various signal transduction inhibitors, including gefinitib and trastuzumab, with paclitaxel and carboplatin have been found to be sequence dependent (24, 25). Therefore, we evaluated the effect of treatment schedule on the efficacy of combination therapy with rapamycin and paclitaxel in vitro. Rapamycin and paclitaxel were given alone or in combination simultaneously. In addition, rapamycin was administered 48, 24, 12, or 6 hours before paclitaxel or, alternately, 6, 12, or 24 hours after paclitaxel administration. The percentage of annexin-positive cells was determined by FACS analysis 48 hours after rapamycin treatment for the rapamycin only-treated group and 48 hours after paclitaxel administration for all of the other treatment groups (Fig. 9A). Compared with cells treated with paclitaxel and rapamycin simultaneously, rapamycin given 12 (P = 0.02) or 24 hours (P = 0.007) after paclitaxel demonstrated significantly more annexin-positive cells, whereas rapamycin treatment 6 hours after paclitaxel administration did not (P = 0.88). When apoptosis was assayed by determining the percentage of cells in sub-G1 with each treatment sequence, the percentage of sub-G1 cells also did not significantly increase com-

Fig. 6  Rapamycin decreases HER2/neu-mediated chemoresistance. NIH3T3 and NIH3T3/HER2 cells were treated with varying concentrations of paclitaxel (left panels) or carboplatin (right panels), in the absence of rapamycin (top panels) or in the presence of 10 or 100 nmol/L rapamycin (middle and bottom panels, respectively). Cell growth inhibition was determined by MTT assay. Cell viability in the rapamycin treatment groups was normalized to their respective no paclitaxel or carboplatin treatment groups to investigate the effect of the chemotherapeutic agents. □, NIH3T3/HER2; ◆, NIH3T3.
pared with simultaneous treatment when rapamycin was given 6 hours after paclitaxel (P = 0.109), but it significantly increased when rapamycin was given 12 (P = 0.009) or 24 hours (P = 0.006) after paclitaxel (Fig. 9B). These results, taken together, demonstrate that rapamycin enhances paclitaxel-induced cytotoxicity in a sequence-dependent manner, and when rapamycin is administered 12 to 24 hours after paclitaxel, apoptosis increases even further, as compared with both agents given simultaneously.

Following the same sequencing schedule, we sought to determine the effect of combination therapy on cell cycle progression. Rapamycin and paclitaxel are known to induce G1 and G2-M cell cycle arrest, respectively. As expected, the percentage of cells in G2-M was increased when MDA-MB-468 cells were treated with paclitaxel alone. In contrast, cells that were pretreated with rapamycin before paclitaxel administration did not have an increase in cells in the G2-M phase (Fig. 9C). However, when paclitaxel was given 6, 12, or 24 hours before rapamycin administration, an increase in cells in G2-M phase was observed, similar to that observed on single-agent paclitaxel therapy. G2-M arrest has been proposed to be a prerequisite step for apoptosis induced by paclitaxel (26); therefore, our results suggest that delayed rapamycin treatment may enhance paclitaxel-induced apoptosis by allowing cells to progress to G2-M arrest. Because our data demonstrated that rapamycin treatment 24 hours after paclitaxel treatment may enhance apoptosis even more than simultaneous treatment, we evaluated the efficacy of these two sequencing schedules on anchorage-dependent growth (Fig. 9D). Cells were treated with paclitaxel alone, rapamycin alone, with the combination simultaneously, or with rapamycin given 24 hours after paclitaxel. Colonies were counted 18 days after treatment. Rapamycin treatment after paclitaxel did demonstrate a statistically significant decrease in clonogenicity when compared with treatment with either agent alone (P < 0.05). Although treatment with rapamycin after paclitaxel resulted in greater inhibition of anchorage-dependent growth when compared with simultaneous treatment, this difference did not reach statistical significance (P = 0.10).

**Rapamycin Enhances the Effect of Chemotherapy In vivo.** We evaluated the effects of rapamycin and paclitaxel, alone and in combination, in nude mice bearing breast cancer xenografts. MDA-MB-468 cells were injected into the mammary fat pads of athymic mice. Sixteen days after inoculation, the mice were divided into six groups and treated with vehicle alone, paclitaxel alone (5 mg/kg), rapamycin alone at one of two doses (1.5 or 15 mg/kg), or a combination of paclitaxel and rapamycin. Treatment was given intraperitoneally once a week for 6 weeks. This in vivo tumor model demonstrated a significant reduction (P < 0.05) in tumor volume on day 28 in mice treated with 15 mg/kg rapamycin in combination with paclitaxel (volume, 32 mm³) compared with that in control mice (232 mm³) and in mice treated with paclitaxel alone (150 mm³) or 15 mg/kg rapamycin alone (110 mm³; Fig. 10A). Similarly, mice treated with paclitaxel plus 15 mg/kg rapamycin showed a significant reduction in tumor volume (29 mm³) compared with the vehicle control (125 mm³; Fig. 10B). These results suggest that rapamycin enhances the effect of chemotherapy in vivo, and that the combination of paclitaxel and rapamycin is more effective than either agent alone.

![Fig 7](image-url) Rapamycin enhances paclitaxel-induced apoptosis in HER2/neu-overexpressing cells. NIH3T3/HER2 (A) and NIH3T3 (B) cells were treated with rapamycin alone (100 nmol/L), paclitaxel alone (1 or 10 µg/mL), or a combination of the two agents for 48 hours. Apoptosis was determined by annexin labeling. Results were reproducible in two other independent experiments.

![Fig 8](image-url) Rapamycin in combination with paclitaxel down-regulates the mTOR signaling pathway. MDA-MB-468 cells were treated with 0.01 µg/mL paclitaxel alone (P), 100 nmol/L rapamycin alone (R), or a combination of the two (PR). After 24 hours, 50 µg of total protein were prepared from cells that were not treated (No Rx) and from each treatment group. Antibodies against phospho-Akt (Ser473), total Akt, phospho-S6K1 (Thr389), total S6K1, phospho-S6 protein, total S6 protein, phospho-4E-BP1 (Ser65), total 4E-BP1, and actin were used for Western blot analysis.
treated with 1.5 mg/kg rapamycin in combination with paclitaxel demonstrated a significant reduction in tumor volume (93 mm³; *P < 0.05*) compared with that seen with paclitaxel alone (150 mm³) and 1.5 mg/kg rapamycin alone (140 mm³). Thus, at the drug dosage used, the treated tumor to control tumor volume ratios were 64.7% for paclitaxel alone, 40.1% for paclitaxel + 1.5 mg/kg rapamycin, and 13.8% for paclitaxel + 15 mg/kg rapamycin. These results demonstrate that rapamycin significantly enhances the efficacy of paclitaxel *in vivo*.

The effect of rapamycin in combination with paclitaxel *in vivo* was found to be reproducible in a second, independent experiment. In this experiment, we also evaluated the effects of altering the sequence of treatments *in vivo*. Mice received injection with 2 × 10⁷ MDA-MB-468 cells in the mammary fat pad. After tumors were established, the mice were treated intraperitoneally with vehicle alone, paclitaxel alone (5 mg/kg), rapamycin alone (15 mg/kg), paclitaxel and rapamycin synchronously (P/R), or rapamycin given 24 hours after paclitaxel (P + 24 hr → R), weekly for 6 weeks. Both the P/R and the P + 24 hr → R groups had a significant reduction in tumor volume when compared with vehicle alone, rapamycin alone, and paclitaxel alone (*P < 0.05*; Fig. 10B). We did not demonstrate a significant reduction in tumor volume in mice treated with rapamycin after paclitaxel compared with mice treated synchronously (*P = 0.15*). Therefore, although there was an increase in apoptosis with sequenced treatment compared with synchronous treatment in our *in vitro* experiments, this did not translate into a significant reduction in tumor volume compared with synchronous treatment *in vivo* in this experiment.

**DISCUSSION**

Rapamycin and rapamycin analogs have emerged as promising antitumor drugs for many cancer types, including breast cancer. The objective of our study was to determine the nature of the interactions between rapamycin and other chemotherapeutic agents to provide guidance regarding how best to combine rapamycin with existing cancer therapies. We found that *in vitro*, rapamycin is synergistic with paclitaxel, carboplatin, and vinorelbine and additive with doxorubicin and gemcitabine. Furthermore, we showed that rapamycin in combination with paclitaxel leads to a significant reduction in tumor growth *in vivo* in a rapamycin-sensitive xenograft model.

PTEN-deficient tumors are thought to be especially sensitive to rapamycin-mediated growth inhibition (27–29). Loss of PTEN and activation of Akt have also been associated with resistance to chemotherapy (30). Recently, Grünwald et al. (31) showed that treatment of PTEN-negative PC-3 prostate cancer cells with rapamycin increased the ability of doxorubicin to kill cells to levels similar to that of PTEN-positive cells. Our results...
Rapamycin Enhances Chemotherapy-Induced Cytotoxicity

**Rapamycin Enhances the Effect of Paclitaxel in vivo**

Treatment with rapamycin and paclitaxel, alone and in combination, was evaluated for in vivo efficacy in a MDA-MB-468 xenograft model. A. Female nude mice received injection in their mammary fat pads with \(1 \times 10^6\) cells per mouse to induce tumor xenografts. Treatments were given once a week for 6 weeks. Groups consisted of vehicle control (No Rx), 5 mg/kg paclitaxel, 1.5 mg/kg rapamycin, 15 mg/kg paclitaxel and 5 mg/kg paclitaxel + 15 mg/kg rapamycin. **P**, paclitaxel; **R**, rapamycin; **P/R**, paclitaxel and rapamycin. **B.** The effect of altering the sequence of treatments was evaluated in vivo. Mice received injection of \(2 \times 10^6\) MDA-MB-468 cells into the mammary fat pad. The mice were treated intraperitoneally with vehicle alone (No Rx), paclitaxel alone (**P**, 5 mg/kg), rapamycin alone (**R**, 15 mg/kg), paclitaxel and rapamycin synchronously (**P/R**), or rapamycin given 24 hours after paclitaxel (**P+24hr-R**), weekly for 6 weeks. Tumor volumes were measured with calipers every other day using the formula \(V = \pi b / 6 \times a^2 \times b\).

**Fig. 10** Rapamycin enhances the effect of paclitaxel in vivo. Treatment with paclitaxel and rapamycin, alone and in combination, was evaluated for in vivo efficacy in a MDA-MB-468 xenograft model. A. Female nude mice received injection in their mammary fat pads with \(1 \times 10^6\) cells per mouse to induce tumor xenografts. Treatments were given once a week for 6 weeks. Groups consisted of vehicle control (No Rx), 5 mg/kg paclitaxel, 1.5 mg/kg rapamycin, 15 mg/kg paclitaxel and 5 mg/kg paclitaxel + 15 mg/kg rapamycin. **P**, paclitaxel; **R**, rapamycin; **P/R**, paclitaxel and rapamycin. **B.** The effect of altering the sequence of treatments was evaluated in vivo. Mice received injection of \(2 \times 10^6\) MDA-MB-468 cells into the mammary fat pad. The mice were treated intraperitoneally with vehicle alone (No Rx), paclitaxel alone (**P**, 5 mg/kg), rapamycin alone (**R**, 15 mg/kg), paclitaxel and rapamycin synchronously (**P/R**), or rapamycin given 24 hours after paclitaxel (**P+24hr-R**), weekly for 6 weeks. Tumor volumes were measured with calipers every other day using the formula \(V = \pi b / 6 \times a^2 \times b\).

suggest that rapamycin-induced chemosensitization is not limited to PTEN-deficient cells because we observed chemosensitization not only in PTEN-negative MDA-MB-468 cells but also in PTEN-positive MCF-7 cells. However, we found that cell lines that are resistant to the growth-inhibitory effect of single-agent rapamycin are also resistant to rapamycin-mediated chemosensitization. Our results are similar to those of Georger et al. (15), who reported that rapamycin did not augment the in vitro cytotoxicity of cisplatin and camptothecin in the rapamycin-resistant primitive neuroectodermal tumor/medulloblastoma cell line D283. We demonstrated previously that breast cancer cells that express high levels of phospho-Akt, independent of PTEN status, and cells that overexpress S6K1 were sensitive to the growth-inhibitory effect of rapamycin (11). These results suggest that phospho-Akt and S6K1 may also help predict which patients will respond to combination therapy with rapamycin.

Amplification or overexpression of the HER2/neu gene occurs in 20% to 30% of human breast cancers and is correlated with poor clinical prognosis (22). Overexpression of HER2/neu has been shown to confer resistance to paclitaxel in breast cancer cells (21). Our data suggest that the mTOR signaling pathway may play an important role in HER2/neu-mediated chemoresistance. Furthermore, our results demonstrate that rapamycin may be able to at least partially overcome resistance to paclitaxel and carboplatin in HER2/neu-overexpressing cells. The recent report (32) that rapamycin reverses chemoresistance in murine lymphomas expressing Akt, but not in those with other apoptotic defects, further emphasizes the importance of selection of tumors that will benefit from rapamycin-mediated chemosensitization. Better elucidation of the mechanism of rapamycin-mediated chemosensitization may help identify additional predictive markers to prospectively identify patients who will benefit the most from this therapeutic approach as well as patients who would benefit from combinations of rapamycin with specific chemotherapy drugs.

One possible mechanism by which rapamycin increases chemosensitivity would be if mTOR acts as a cell survival molecule. In support of this hypothesis, Wan and Helman (33) showed that insulin-like growth factor II protects myoblasts from cisplatin-induced apoptosis and that blockade of S6K1 activity with rapamycin abrogates insulin-like growth factor II-induced protection. S6K1 has been found to phosphorylate and inactivate the proapoptotic molecule BAD, a process inhibited by rapamycin (34). It is interesting that the antitumor effects of both paclitaxel and the platinum agent cisplatin have also been proposed to be mediated, at least in part, by inhibition of S6K1 (23, 33). When phosphorylated, the other downstream target of mTOR, 4E-BP1, releases the cap-binding protein eIF4E from a functionally inactive complex, enabling it to activate translation. eIF4E can substitute for serum and growth factors in preserving the viability of NIH3T3 cells, and overexpression of 4E-BP1 phosphorylation site mutants triggers apoptosis (35, 36). Furthermore, 4E-BP1 itself undergoes caspase-dependent cleavage in cells undergoing apoptosis, leading to an NH\(_2\)-terminally truncated polypeptide that fails to become highly phosphorylated and dissociate from eIF4E (37). Thus, initiation of apoptosis by cytotoxic agents may enhance the effect of rapamycin by further decreasing the availability of eIF4E. These results, taken together, suggest that mTOR and downstream effectors of mTOR, S6K1 and eIF4E, play a role in cell survival and that inhibition of mTOR in the setting of cytotoxic injury may substantially potentiate apoptosis.

Paclitaxel and platinum agents have been shown to activate c-Jun NH\(_2\)-terminal kinase (JNK), and this is thought to contribute to the cell death induced by these agents (38–41). Rapamycin has also been shown to induce sustained activation of apoptosis signal-regulating kinase 1 and JNK and elevation of phosphorylated c-jun, resulting in apoptosis under serum-free conditions (42) Thus, one could speculate that rapamycin in combination with chemotherapeutic drugs may further potentiate activation of the JNK signaling pathway. P53 suppresses the rapamycin-induced sustained activation of the JNK cascade and apoptosis, which is consistent with the finding that rapamycin induces apoptosis in cells lacking functional p53, whereas cells expressing wild-type p53 arrest in the G\(_1\) phase (12, 13, 42). We have found that rapamycin enhanced chemotherapy-induced
apoptosis in both p53 mutant MDA-MB-468 cells and p53 wild-type MCF-7 cells, indicating that the effect of rapamycin on chemotherapy-induced cytotoxicity is not limited to p53 mutant cells. However, because our work was not conducted with isogenic cell lines, we cannot exclude the possibility that the chemosensitizing effect of rapamycin may be more prominent in cells lacking functional p53.

Taken together, our results demonstrate that rapamycin potentiates the cytotoxicity of selected chemotherapeutic agents, especially paclitaxel, carboplatin, and vinorelbine. Our in vitro data suggest that this effect may be sequence dependent; however, further work is needed to determine whether the therapy sequence alters in vivo efficacy. Combination therapy may be especially effective in appropriately selected patients. Determination of the mechanism of rapamycin-mediated chemosensitization is necessary to help identify patients who will benefit the most from this therapeutic approach.

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Targeting Mammalian Target of Rapamycin Synergistically Enhances Chemotherapy-Induced Cytotoxicity in Breast Cancer Cells

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