# Cyclin D3 is down-regulated by rapamycin in HER-2-overexpressing breast cancer cells

**Pilar García-Morales,[1] Eva Hernando,[3] Estefanía Carrasco-García,[1] María Piedad Menéndez-Gutierrez,[1] Miguel Saceda,[1,2] and Isabel Martínez-Lacaci[1]**

[1]Instituto de Biología Molecular y Celular, Universidad Miguel Hernández; [2]Unidad de Investigación, Hospital General Universitario de Elche, Elche, Spain and [3]Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, New York

## Abstract

**Rapamycin and its analogues are being tested as new antitumor agents. Rapamycin binds to FKBP-12 and this complex inhibits the activity of FRAP/mammalian target of rapamycin, which leads to dephosphorylation of 4EBP1 and p70 S6 kinase, resulting in blockade of translation initiation. We have found that RAP inhibits the growth of HER-2-overexpressing breast cancer cells. The phosphorylation of mammalian target of rapamycin, p70 S6 kinase, and 4EBP1 is inhibited by rapamycin and cells are arrested in the $G_1$ phase, as determined by growth assays, fluorescence-activated cell sorting analysis, and bromodeoxyuridine incorporation studies. Rapamycin causes down-regulation of cyclin D3 protein, retinoblastoma hypophosphorylation, loss of cyclin-dependent kinase (cdk) 4, cdk6, and cdk2 activity. The half-life of cyclin D3 protein decreases after rapamycin treatment, but not its synthesis, whereas the synthesis or half-life of cyclin D1 protein is not affected by the drug. Additionally, rapamycin caused accumulation of ubiquitinated forms of cyclin D3 protein, proteasome inhibitors blocked the effect of rapamycin on cyclin D3, and rapamycin stimulated the activity of the proteasome, showing that the effect of rapamycin on cyclin D3 is proteasome proteolysis dependent. This effect depends on the activity of HER-2 because Herceptin, a neutralizing antibody against HER-2, is able to block both the induction of proteasome activity and the cyclin D3 down-regulation due to rapamycin. Furthermore, inhibition of *HER-2* gene expression by using small interfering RNA blocked the rapamycin effects on cyclin D3. These data indicate that rapamycin causes a $G_1$ arrest in HER-2-overexpressing breast cancer cells that is associated with a differential destabilization and subsequent down-regulation of cyclin D3 protein. [Mol Cancer Ther 2006;5(9):2172–81]**

## Introduction

Rapamycin is a macrolide antibiotic that inhibits the growth of yeast and several mammalian cells, including B and T cells and tumor cells. Rapamycin is a potent immunosuppresant that is being currently used as a potential antitumor agent (1). Rapamycin causes a $G_1$ arrest in several tumor types. However, the nature of the $G_1$ block varies depending on the cell type. Rapamycin binds to FKBP-12 and this complex inhibits the activity of FRAP/mammalian target of rapamycin (mTOR), a serine/threonine kinase that acts downstream of phosphatidylinositol 3-kinase (PI3K) and Akt (2). Inhibition of mTOR leads to dephosphorylation of 4EBP1 (3) and its consequent binding to eIF4E (4) and to inactivation of p70 S6 kinase ($p70^{S6k}$; ref. 5), resulting in inhibition of translation of mRNAs with a highly structured 5′-coding region or with a 5′-oligopyrimidine tract, respectively, most of them components of the translational machinery (6).

HER-2 amplification and/or overexpression occurs in 25% to 30% of human breast cancer and is correlated with poor prognosis (7). HER-2/HER-3 heterodimers constitutively activate PI3K in breast cancer cell lines with HER-2 overexpression or gene amplification (8). Activation of PI3K and phosphatidylinositol generation are required for activation of Akt and $p70^{S6k}$ (9). Cyclin D expression is dependent on the PI3K/Akt pathway in breast cancer cells (10). D-type cyclins and their associated kinases act as sensors of the external stimuli elicited by transmembrane receptors that allow cells to progress through the $G_1$ phase of the cell cycle (11). Cyclin D–cyclin-dependent kinases (cdk) 4 and cdk6, cyclin E-cdk2, and cyclin A-cdk2 act sequentially in the progression through the $G_1$ and S phases (12). Cdk activities are regulated by the Cip/Kip (p21, p27, and p57) and INK (p15, p16, p18, and p19) families of cdk inhibitors and phosphorylate retinoblastoma, which is the limiting factor in the $G_1$-S transition. When retinoblastoma is phosphorylated, it releases E2F factors allowing S phase progression (13). Blocking mTOR with rapamycin, which acts downstream of Akt, could inhibit growth of breast cancer cells that express high levels of HER-2 by inducing a $G_1$ arrest of the cell cycle.

We wanted to study the effect of rapamycin in breast cancer cell lines, particularly in SKBr-3, BT-474, MDA-453, and MDA-361 cells, which are known to possess *HER-2*

Received 9/9/05; revised 6/23/06; accepted 7/12/06.

**Grant support:** Instituto de Salud Carlos III grant FIS PI041084, Generalitat Valenciana grant GV04B-669, and Ministerio de Ciencia y Tecnología grant RYC-2002-002243 (I. Martínez-Lacaci).

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**Requests for reprints:** Isabel Martínez-Lacaci, Instituto de Biología Molecular y Celular, Edificio Torregaitán, Universidad Miguel Hernández, 03202 Elche, Spain. Phone: 34-96-6658744; Fax: 34-96-6658758. E-mail: imlacaci@umh.es



doi:10.1158/1535-7163.MCT-05-0363

gene amplification and overexpression (14), and in which the PI3K/Akt/mTOR pathway is activated by HER-2/HER-3 heterodimers (15). We believe that by inhibiting this pathway cell growth will be inhibited. Indeed, our data indicate that HER-2-overexpressing cancer cells were very sensitive to rapamycin. These cells were arrested in $G_1$ and, interestingly, the main effect was seen on cyclin D3 down-regulation but not in cyclin D1. Rapamycin affected the stability of cyclin D3 by activating the proteasome-ubiquitin pathway and this effect was also dependent on HER-2 activation. Taken together, these data suggest that breast cancer cells with gene amplification or overexpression of HER-2 are very responsive to rapamycin, which opens a myriad of possibilities to treat breast tumors with rapamycin derivatives, such as CCI-779 (Wyeth-Ayerst Laboratories, Madison, NJ), RAD001 (Novartis, Basel, Switzerland), or AP23573 (16), which have shown promising activity in early clinical trials with low toxicity profiles and are currently undergoing phase II and III trials on patients with solid tumors and some hematologic malignancies (17, 18). Likewise, breast cancer patients with HER-2 amplifications would be susceptible for targeted therapies using rapamycin derivatives as we show in this report.

## Materials and Methods

### Reagents

Rapamycin was purchased from Sigma (St. Louis, MO) and dissolved in DMSO. Propidium iodide and bromodeoxyuridine (BrdUrd) were purchased from Sigma. LY294002 was purchased from Biomol (Plymouth Meeting, PA) and dissolved in DMSO. Proteasome inhibitors (lactacystin, MG132, and proteasome inhibitor I) and the proteasome activity assay kit were purchased from Calbiochem (San Diego, CA). *Neu* small interfering RNA (siRNA; h) and control siRNA-A were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Herceptin (trastuzumab) was purchased from Roche Farma S.A. (Madrid, Spain).

### Cell Culture

The human breast cancer cell lines SKBr-3, BT-474, MDA-MB-453 (MDA-453), MDA-MB-361 (MDA-361), MDA-MB-468 (MDA-468), MCF-7, and BT-549 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM-F12 supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, and 50 units/mL penicillin and 50 μg/mL streptomycin and incubated at 37°C in a humidified 5% $CO_2$/air atmosphere.

### Cell Proliferation Assays

Cells were plated in 96-well plates, treated with vehicle (DMSO) or rapamycin at different times and concentrations, stained with Alamar Blue (Accumed, Westlake, OH) for 4 hours, and analyzed with a SpectraMax Gemini XS microplate fluorometer (excitation, 530 nm; emission, 590 nm; Molecular Devices, Sunnyvale, CA) as described previously (19).

### Flow Cytometric Analysis of Cell Cycle Distribution

Cells were plated and treated with rapamycin for particular times or concentrations. Cells were trypsinized and washed with PBS and nuclei were isolated as described previously (20), stained with ethidium bromide, and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Mansfield, MA). To determine cell cycle distribution of DNA content, Multicycle program software (Phoenix Flow Systems, San Diego, CA) was utilized.

### BrdUrd Incorporation

Cells were plated and treated with DMSO or rapamycin as mentioned above. Cells were pulsed with BrdUrd for 1 hour before harvesting and then trypsinized, washed with PBS, and fixed with 80% ethanol. Subsequently, DNA was denatured with 2 N HCl/0.5% Triton X-100/PBS, blocked with PBS containing 1% bovine serum albumin, and incubated for 30 minutes at room temperature in the dark with an anti-BrdUrd antibody conjugated to FITC or the isotypic control antibody-FITC (BD PharMingen, San Diego, CA). Cells were washed, incubated with PBS containing propidium iodide (5 μg/mL) and RNase A to counterstain DNA, and analyzed by flow cytometry using CellQuest software (Becton Dickinson).

### Western Blot Analysis

Cells were plated, treated with DMSO or rapamycin, washed with PBS, and lysed in a lysis buffer containing 50 mmol/L Tris (pH 7.4), 1% NP40, 150 mmol/L NaCl, 30 mmol/L sodium pyrophosphate, 40 mmol/L NaF, 1 mmol/L $Na_3VO_4$, 5 mmol/L β-glycerophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 μg/mL each of leupeptin, aprotinin, and soybean trypsin inhibitor for 30 minutes on ice. Supernatants were cleared by centrifugation and protein concentration was determined by the protein assay reagent method (Pierce Chemical Co., Rockford, IL). Then, 50 to 80 μg protein from lysates were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, incubated with primary antibodies against cyclin D3, cyclin D1, cdk4, cdk6, cdk2, 4EBP1, $p27^{kip1}$, $p21^{cip1}$, c-*neu* (HER-2; Santa Cruz Biotechnology), phosphorylated mTOR (S2448), mTOR, phosphorylated $p70^{S6k}$ (T421/S424), $p70^{S6k}$, phosphorylated 4EBP1 (S65; Cell Signaling Technology, Beverly, MA), retinoblastoma (BD PharMingen), or actin (Sigma), washed, and incubated with horseradish peroxidase–linked secondary antibodies. Membranes were washed and proteins were detected by the enhanced chemiluminescence method (Amersham, Piscataway, NJ). Densitometric analyses were done with the Bio-Rad Gel Doc system (Bio-Rad, Hercules, CA).

### Immunoprecipitation

Cells were treated, harvested, and lysed as above. Then, 500 to 700 μg protein were incubated with antibodies against cdk4, cdk6 (Neomarkers, Lab Vision Corp., Union City, CA), or cdk2 (Santa Cruz Biotechnology) and rotated end over end overnight at 4°C followed by incubation for 1 hour at 4°C with protein G-Sepharose beads (Pharmacia-Amersham Biosciences, Piscataway, NJ). Beads were washed four times with lysis buffer and resuspended in SDS-PAGE sample buffer. Proteins were eluted by boiling

the beads and subjected to SDS-PAGE electrophoresis, transferred to polyvinylidene difluoride membranes, incubated with primary antibodies, washed, incubated with secondary antibodies, incubated with enhanced chemiluminescence reagents, and exposed to autoradiography.

### Immunocomplex *In vitro* Kinase Assays

Cells were treated as above, lysed in lysis buffer, and sonicated, and supernatants were cleared by centrifugation. Protein (100 μg) was incubated with cdk4 or cdk6 antibodies (Neomarkers, Lab Vision, Fremont, CA) overnight at 4°C and rotated end over end. Subsequently, immunocomplexes were incubated for 1 hour at 4°C with protein G-Sepharose, washed twice with lysis buffer, washed twice with kinase buffer (250 mmol/L HEPES-KOH, 50 mmol/L $MgCl_2$, 5 mmol/L DTT, 12.5 mmol/L EGTA, 50 mmol/L β-glycerophosphate, 5 mmol/L NaF, 0.5 mmol/L $Na_3OV_4$), and incubated in kinase buffer containing 0.2 μg glutathione *S*-transferase–retinoblastoma (Cell Signaling Technology), 10 μCi [$\gamma$-$^{32}$]ATP, and 300 μmol/L ATP for 20 minutes at 30°C. Alternatively, 100 μg protein lysates were incubated by rotating end over end with a cdk2 antibody (Santa Cruz Biotechnology) overnight at 4°C and incubated for 1 hour at 4°C with protein G-Sepharose. Beads were washed twice with lysis buffer and twice with kinase buffer [20 mmol/L Tris (pH 7.4), 7.5 mmol/L $MgCl_2$, 1 mmol/L DTT] and incubated in kinase buffer containing 2 μg histone H1 (Upstate Biotechnology, Lake Placid, NY), 10 μCi [$\gamma$-$^{32}$]ATP, and 300 μmol/L ATP for 20 minutes at 37°C. The reactions were stopped by addition of SDS-PAGE sample buffer and boiled. Proteins were resolved on SDS-PAGE, transferred onto polyvinylidene difluoride, and exposed to autoradiography.

### Proteasome Activity Assay

Cells were plated and treated as indicated above. Cells were harvested and incubated with a buffer containing 10 mmol/L Tris (pH 7.5), 1 mmol/L EDTA, and 20% glycerol for 15 minutes on ice and centrifuged at 13,000 × *g* for 10 minutes at 4°C, and protein concentration was determined. Then, protein extracts (10 μg) were incubated in a reaction buffer containing SDS and a fluorogenic substrate according to the manufacturer's instructions and fluorescence was determined in a microplate fluorometer (Fluostar Galaxy; excitation, 370 nm; emission, 460 nm). As a positive control, the 20S proteasome purified enzyme was used, and negative controls included protein extracts and the purified enzyme incubated with proteasome inhibitors.

### siRNA-Mediated Inhibition of *HER-2* Gene Expression

Cells were plated in 35-mm dishes, transfected with HER-2 siRNA or control siRNA (scrambled sequence) according to the manufacturer's instructions, and treated with DMSO or rapamycin for 16 hours. Cells were harvested, lysed, and subjected to Western blot analyses.

## Results

### Rapamycin Effects on Cell Growth and Cell Cycle Distribution

SKBr-3 cells were growth inhibited with concentrations starting from 1 nmol/L rapamycin (Fig. 1A). Growth inhibitory effects in other HER-2-overexpressing cell lines (BT-474, MDA-453, and MDA-361) and non–HER-2-overexpressing cell lines (MCF-7, MDA-468, and BT-549) were similar to the effects seen in SKBr-3 cells, with $IC_{50}$ ranging from 0.1 to 10 nmol/L (data not shown). Cells did not undergo apoptosis, as revealed by flow cytometry and Hoechst staining (data not shown). We did cell cycle analysis (Fig. 1B) and found that SKBr-3 cells were arrested in $G_1$ after treating them with 10 nmol/L rapamycin for 24 hours and that this block was sustained for at least 72 hours (Fig. 1C). BrdUrd incorporation studies confirmed that SKBr-3 treated with rapamycin did not enter into S phase (Fig. 1D) and at the same time, the percentage of cells in the S phase was smaller in the rapamycin-treated cells than in the DMSO-treated cells (Fig. 1E). Same effects were observed in other HER-2-overexpressing cells (BT-474, MDA-453, and MDA-361), whereas non–HER-2-overexpressing cells (MDA-468 and BT-549) did not show a very pronounced $G_1$ arrest (Supplementary Fig. S1).[4]

### Rapamycin Effects on Immediate Targets

To investigate the effects of rapamycin on its immediate targets, SKBr-3 cells were treated with vehicle (DMSO) or 10 nmol/L rapamycin for different times and subjected to Western blot analysis using the following antibodies: phosphorylated mTOR, mTOR, phosphorylated $p70^{S6k}$, $p70^{S6k}$, phosphorylated 4EBP1, 4EBP1, phosphorylated Akt, and Akt (Fig. 2). mTOR phosphorylation (S2448) decreased after rapamycin treatment at all the times analyzed. Phosphorylation of the two S6k isoforms, p70 and p85, disappeared using a phosphorylated antibody (T421/S424) and a shift to lower molecular weight band of both p85 and p70 isoforms was observed with a total $p70^{S6k}$ antibody. 4EBP1 phosphorylation (S65) was also affected and a band shift using an antibody against total 4EBP1 was appreciated with rapamycin. Akt phosphorylation and protein levels were unaltered at any time or concentration with rapamycin because Akt acts upstream of mTOR.

### Effect of Rapamycin on D-Cyclins and Retinoblastoma

To investigate the nature of the cell cycle arrest induced by rapamycin, the levels of cell cycle proteins involved in regulation of the $G_1$ phase, particularly D-cyclins and retinoblastoma, were analyzed. Steady-state levels of cyclin D3 decreased at a greater extent than cyclin D1, and retinoblastoma underwent hypophosphorylation in SKBr-3 cells after rapamycin treatment (Fig. 3A and B). The decrease in cyclin D3 steady-state levels was also observed in the other HER-2-overexpressing BT-474, MDA-453, and MDA-361 cells, but not in MDA-468 or MCF-7 breast cancer cells, which do not overexpress HER-2 (Fig. 3C). We tested the effect of rapamycin on glioblastoma, prostate, and pancreatic carcinoma cell lines, which do not overexpress HER-2 and did not found this differential effect on cyclin D3 down-regulation (data not shown). Next, we wanted to determine whether the selective effect of rapamycin on

[4] Supplementary materials for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

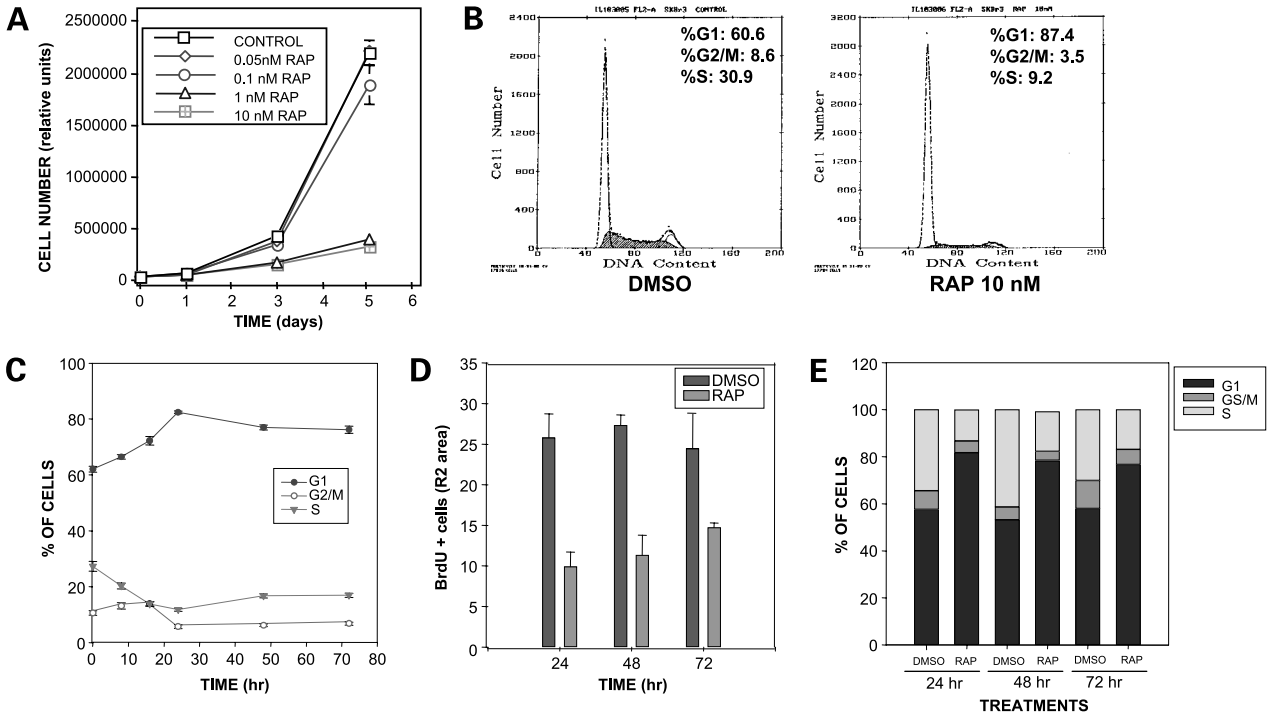


**Figure 1.** Effects of rapamycin (*RAP*) on cell proliferation. **A,** SKBr-3 were grown in 96-well plates, treated with DMSO or rapamycin at the times and concentrations indicated, and incubated with Alamar Blue and cell proliferation rates were determined by fluorescence. Effects of rapamycin on cell cycle. SKBr-3 cells were plated and treated with DMSO or 10 nmol/L rapamycin for 24 h. Cell cycle distribution of DNA content was determined by flow cytometry as described in Materials and Methods. **B,** SKBr-3 cells treated with DMSO or 10 nmol/L rapamycin for 24 h. **C,** time course of cell cycle distribution after rapamycin treatment. SKBr-3 cells were plated and treated with DMSO (time 0) or 10 nmol/L rapamycin for the times indicated, and cell cycle distribution was determined by flow cytometry and represented as the average of six separate experiments. BrdUrd incorporation and cell cycle analysis. SKBr-3 were plated and treated with DMSO or 10 nmol/L rapamycin for 24, 48, and 72 h. **D,** DNA synthesis was determined by BrdUrd incorporation. **E,** the percentage of positive cells were represented as the average of three experiments, and at the same time, cell cycle analysis was done in the same experiments as described in Materials and Methods.

cyclin D3 protein was exclusive of rapamycin and not of other inhibitors of the PI3K/Akt/mTOR pathway. We treated SKBr-3 cells with DMSO or the PI3K inhibitor LY294002. The decrease in cyclin D1 was much faster in this case than the reduction of cyclin D3 levels (Fig. 3B) and retinoblastoma was also hypophosphorylated. Cyclin D-associated activities diminished after rapamycin treatment in SKBr-3 cells, as revealed by *in vitro* kinase assays done by immunoprecipitating cdk4 and cdk6 proteins. However, the cdk4 and cdk6 protein levels were not affected by rapamycin. Additionally, the association of cdk4 with cyclin D3 decreased after rapamycin treatment, whereas the association of cdk4 with cyclin D1 was less affected by rapamycin (Fig. 4A), paralleling the drug effect in cyclin D1 and D3 proteins.

### Rapamycin Inhibits Cdk2 Kinase Activity

Rapamycin induces $G_1$ arrest and hypophosphorylation of retinoblastoma in human breast cell lines that overexpress HER-2, which is associated with cyclin D3 down-regulation. The effect of rapamycin on cdk2 and its regulatory subunits was further investigated. The activity of cdk2 was inhibited with rapamycin in SKBr-3 cells (Fig. 4B), but the levels of cdk2 protein remained constant. The levels of cyclin E and cyclin A were unaffected by rapamycin as well (Fig. 4B).

### D-Cyclin mRNA Is Not Affected by Rapamycin

Rapamycin causes down-regulation of cyclin D3 but not cyclin D1. To determine whether the decrease in protein levels is due to a reduction of mRNA levels, we analyzed the levels of cyclin D1 mRNA and cyclin D3 mRNA by semiquantitative reverse transcription-PCR after treating SKBr-3 cells with rapamycin for several times (Supplementary Fig. S2).[4] D-cyclin mRNA levels did not change after rapamycin treatment at any time. However, as we have already shown (Fig. 3A and B), the cyclin D3 steady-state protein levels experience a marked decrease.

### Rapamycin Affects the Half-life of Cyclin D3 Protein

To determine whether the reduction of cyclin D3 protein levels was caused by a decrease in protein synthesis, a decrease in protein half-life or both, we determined the rate of incorporation of radiolabeled amino acids into cyclin D1 and cyclin D3 in the presence or absence of rapamycin for the times indicated (Supplementary Fig. S3).[4] The rate of synthesis of either cyclin D1 or cyclin D3 was unaffected by the drug treatments. Next, we determined the half-life of cyclin D1 and cyclin D3 protein by treating SKBr-3 cells with cycloheximide for different times in the presence or absence of rapamycin. Cyclin D1 half-life is shorter than cyclin D3 half-life and it was not affected by rapamycin (Fig. 5). However,

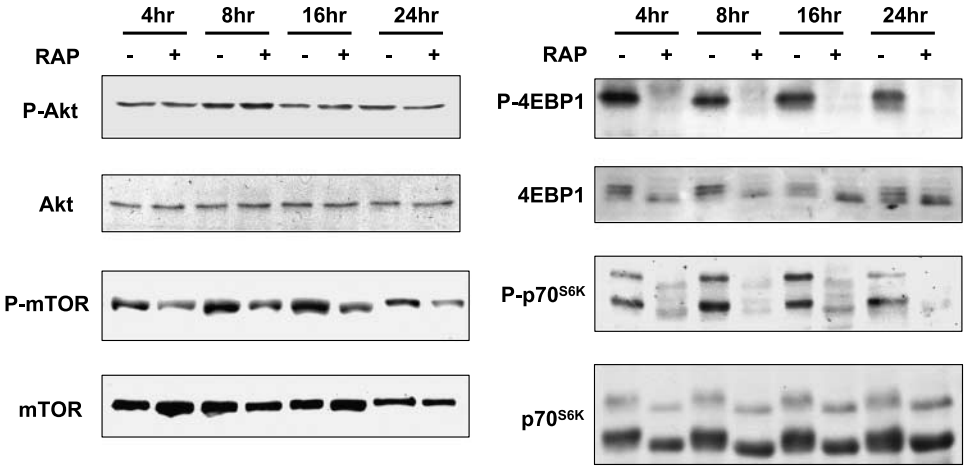


**Figure 2.** Effects of rapamycin on immediate targets. SKBr-3 cells were grown and treated with DMSO or 10 nmol/L rapamycin for 4, 8, 16, and 24 h and subjected to Western blot analysis using the antibodies indicated.

rapamycin decreased the half-life of cyclin D3 protein from 1.5 to 0.5 hours, indicating that rapamycin affects cyclin D3 expression by reducing its half-life and probably making it less stable. We obtained similar results with pulse-chase experiments (data not shown), confirming these findings.

### Rapamycin Induces Ubiquitination of Cyclin D3 Protein

D-cyclins, as many cell cycle regulatory proteins, are targeted to degradation by the proteasome-ubiquitin pathway. SKBr-3 cells were left untreated or treated with rapamycin. Cyclin D3 was immunoprecipitated and its association with ubiquitin was determined by Western blot, finding ubiquitinated forms of cyclin D3 protein after treatment with rapamycin (Fig. 6A).

### Proteasome Inhibitors Abolish the Rapamycin Effect on Cyclin D3 Protein

To confirm that rapamycin affects the stability of cyclin D3 protein rather that its *de novo* synthesis, SKBr-3 cells

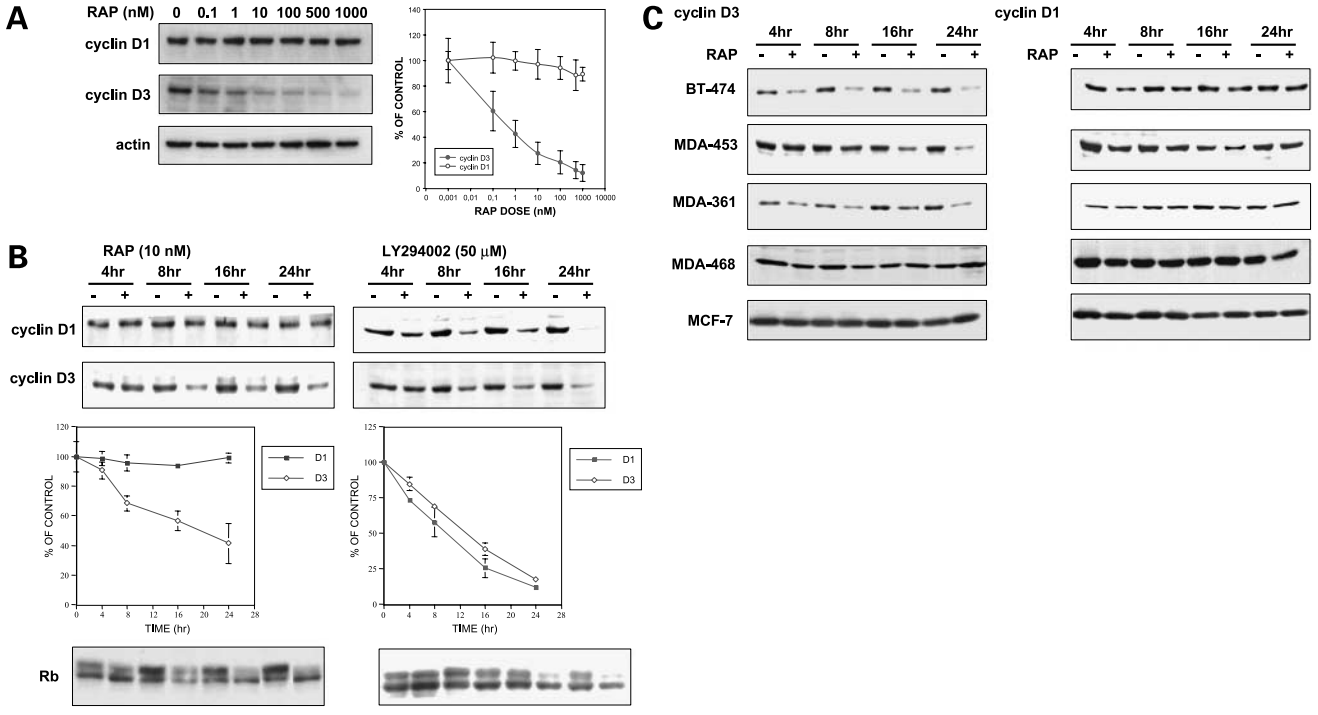


**Figure 3.** Effects of rapamycin and LY294002 on D-cyclins and retinoblastoma. **A,** SKBr-3 cells were treated with DMSO or different concentrations of rapamycin for 24 h and the levels of cyclin D1, cyclin D3, and actin were determined by Western blot. Protein bands were quantified and represented as the average of four separate experiments. **B,** SKBr-3 cells were treated with DMSO, 10 nmol/L rapamycin, 50 μmol/L LY294002 for 4, 8, 16, and 24 h and the levels of cyclin D1, cyclin D3, and retinoblastoma were determined by Western blot. Protein bands were quantified and represented as the average of six separate experiments. **C,** BT-474, MDA-453, MDA-361, MDA-468, and MCF-7 cells were treated with DMSO or 10 nmol/L rapamycin for 4, 8, 16, and 24 h and the levels of cyclin D3 and cyclin D1 proteins were determined by Western blot.

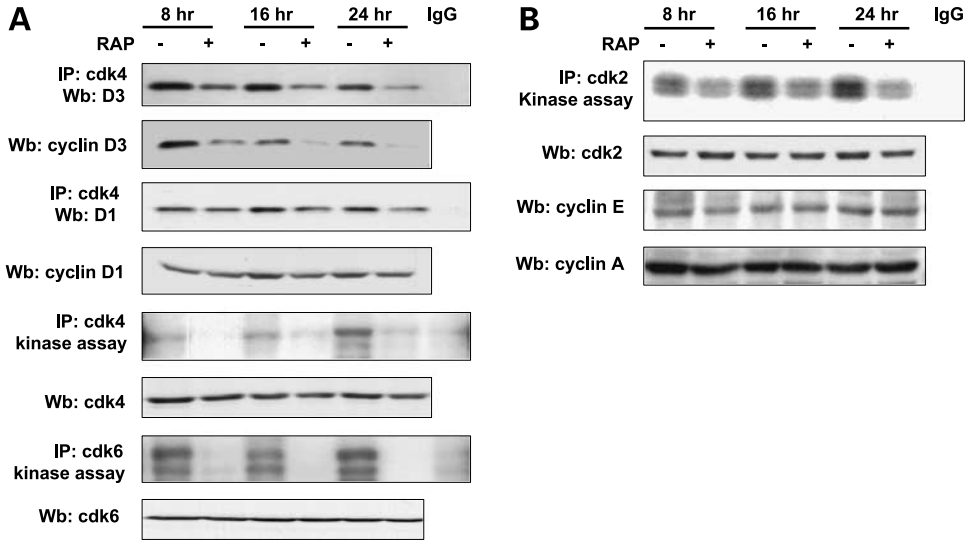


**Figure 4.** Effect of rapamycin on cdk4 and cdk6 activities. **A**, SKBr-3 cells were treated with DMSO or 10 nmol/L rapamycin for the times indicated. Cdk4 protein was immunoprecipitated (*IP*) and its association with cyclin D3 and cyclin D1 was determined by Western blot (*Wb*). Protein total levels of cyclin D3 and cyclin D1 from the same samples were analyzed by Western blot. *In vitro* kinase assays were done with antibodies against cdk4 and cdk6. The total levels of these proteins were analyzed by Western blot. Effect of rapamycin on cdk2 activity. **B**, SKBr-3 cells were treated with DMSO or 10 nmol/L rapamycin for the times indicated. *In vitro* kinase assays were done with antibodies against cdk2. Total protein levels of cdk2, cyclin E, and cyclin A were analyzed by Western blot.

were treated with rapamycin in the presence or absence of proteasome inhibitors, such as lactacystin, MG132, and proteasome inhibitor I. The three proteasome inhibitors were able to abolish the reduction of cyclin D3 protein caused by rapamycin (Fig. 6B). The protein levels of cyclin D1 and other cell cycle regulatory proteins, such as $p27^{kip1}$or $p21^{cip1}$ (included as controls), were also augmented after treatment with proteasome inhibitors.

### Rapamycin Induction of Proteasomal Activity Depends on HER-2 Expression and Activity

Cyclin D3 down-regulation due to rapamycin was more evident in HER-2-overexpressing cells than in other breast cancer cells that do not overexpress HER-2, such as MDA-468 and MCF-7 cells (Fig. 3). To test whether the activation of HER-2 was related to cyclin D3 down-regulation, SKBr-3 cells were treated with rapamycin in the presence or absence of Herceptin (trastuzumab), a neutralizing antibody against HER-2. Herceptin was able to restore cyclin D3 protein levels almost to control levels, abolishing, at least partially, the rapamycin effect (Fig. 6C). By contrast, Herceptin had no effect on cyclin D1 protein levels (Fig. 6C). We wanted to determine the activity of the proteasome under the same conditions. Treatment with 10 nmol/L rapamycin produced a 3-fold induction of proteasome activity and this effect was abolished by Herceptin (Fig. 6D), indicating that the effect of rapamycin on the induction of the proteasome-ubiquitin pathway is dependent on the activity of HER-2 in SKBr-3 cells. To determine whether the HER-2 expression was related to cyclin D3 down-regulation, SKBr-3 cells were transfected with HER-2 siRNA and control siRNA before treating them with DMSO or rapamycin. The HER-2 expression levels were reduced in the HER-2 siRNA–transfected cells compared with cells transfected with a scrambled sequence of siRNA and the cyclin D3 levels were partially restored after rapamycin treatment in the HER-2 siRNA–transfected cells (Fig. 6E), indicating that the effect of rapamycin on cyclin D3 down-regulation depends on the HER-2 expression levels.

## Discussion

It has been shown that human breast cancer cells that overexpress HER-2 require the PI3K and mTOR/$p70^{S6k}$ pathway for anchorage-independent growth (21). The mTOR inhibitor rapamycin has been shown to block focus formation induced by oncogenic alleles of PI3K and Akt (22). In addition, cyclin D is post-transcriptionally modulated by the PI3K/Akt pathway (10). Rapamycin inhibits the $G_1$-S transition by blocking the induction of cyclin D1 in serum-stimulated NIH3T3 cells (23), in human pancreatic cells (24), and in renal cancer cells (25). In this study, we set out to determine the effect of rapamycin in human breast cancer cells that overexpress HER-2 (14) and have the PI3K/Akt/mTOR pathway overactivated (15). This report is the first evidence suggesting that HER-2 plays a role in the regulation of cyclin D3.

We show that HER-2-overexpressing cells (SKBr-3, BT-474, MDA-453, and MDA-361) are sensitive to rapamycin and are arrested in $G_1$ (Fig. 1). This $G_1$ arrest is sustained, whereas, in MDA-468 cells, which do not overexpress HER-2, the $G_1$ arrest is not maintained (data not shown). Other reports have shown a $G_1$ arrest in different types of cells due to rapamycin (26, 27). The growth-inhibitory effects of rapamycin and its derivative CCI-779 in a panel of different breast cancer cell lines have been investigated by other groups (28, 29), showing similar results to the ones presented herein. Rapamycin inhibited phosphorylation of mTOR, $p70^{S6k}$, and 4EBP1 (Fig. 2), but not of Akt, which is upstream of mTOR. This indicates that inhibition of mTOR is not equivalent to inhibition of Akt, which impinges in targets different than mTOR targets (i.e., GSK-3, FKHRL-1, and BAD). The retinoblastoma hypophosphorylation that takes places after rapamycin treatment (Fig. 3B) is associated with the $G_1$ arrest and D-cyclin down-regulation. Interestingly, cyclin D3 is affected by the drug in a dose- and time-dependent manner, whereas cyclin D1 is not (Fig. 3). The $IC_{50}$ of cyclin D3 down-regulation is between 0.1 and 1 nmol/L,

paralleling the $IC_{50}$ of growth inhibition (Fig. 1A). The cyclin D3 down-regulation is more prominent in HER-2-overexpressing cells than in other breast cancer cells that do not overexpress HER-2, such as MCF-7 or MDA-468 cells (Fig. 3C), and other tumor cells from disparate tissues. A different study shows that overexpression of $p70^{S6k}$, Akt phosphorylation, and changes in cyclin D1 may be predictors of rapamycin sensitivity (29). However, the changes in cyclin D1 that they observed in some of the breast cancer cell lines took place after treating the cells with 100 nmol/L rapamycin for as long as 4 days. We see changes in cyclin D3 protein levels with only 10 nmol/L rapamycin and after 8 or 16 hours treatment in HER-2-overexpressing cells. In cells that do not overexpress HER-2, these changes may occur at a later time and at higher drug concentrations. The most striking finding of this report is the differential effect of rapamycin on cyclin D3 down-regulation, effect that has not been observed in this system with PI3K inhibitors, such as LY294002 (Fig. 3). An effect of rapamycin on cyclin D3 has been reported in spermatogonia (30), B-CLL cells (31), T lymphocytes (32, 33), and renal epithelial cells (34) but under completely different conditions. The decrease of cdk4- and cdk6-associated activities occurs probably as a consequence of the cyclin D3 loss (Fig. 4A). In addition, the levels of cdk2 activity, which is after cdk4/cdk6 activation, also decreased on rapamycin treatment (Fig. 4B). However, we have not been able to detect any change in cdk2, cyclin E, or cyclin A protein levels after rapamycin treatment in our system.

To determine the mechanism of cyclin D3 down-regulation, the levels of mRNA were analyzed by semi-quantitative reverse transcription-PCR and we found that levels of mRNA were not affected by rapamycin (Supplementary Fig. S2), indicating a post-transcriptional mechanism. The steady-state levels of cyclin D3 protein are reduced 30% within 8 hours, whereas the steady-state levels of cyclin D1 protein are unaltered (Fig. 3). The rate of synthesis of both cyclin D1 and cyclin D3 protein was not affected by the drug (Supplementary Fig. S3). The half-life of cyclin D1 protein is ~40 minutes and is not affected by rapamycin (Fig. 5). However, the half-life of cyclin D3 protein decreases from 1.5 hours to 30 minutes after rapamycin treatment. Rapamycin seems to make the protein less stable and more susceptible for degradation by the ubiquitin-proteosome pathway. In fact, we were able to detect ubiquitinated forms of cyclin D3 protein (Fig. 6A) and proteasome inhibitors were able to abolish the rapamycin effect on cyclin D3 (Fig. 6B), suggesting that the down-regulation of cyclin D3 is proteasome proteolysis dependent. Furthermore, the activity of the proteasome was increased after rapamycin treatment (Fig. 6D), indicating that cyclin D3 and most probably other proteins as well may be affected by this augmented activity of the proteasome. When Herceptin was used, the activity of the proteasome decreased to control levels (Fig. 6D), indicating a clear relationship between HER-2 overexpression and proteasome activity. The effect of Herceptin on rapamycin-driven cyclin D3 down-regulation was investigated and found that Herceptin was also able to abolish, at least partially, the down-regulation of cyclin D3 after rapamycin treatment, whereas cyclin D1 protein levels were not affected (Fig. 6C). Furthermore, *HER-2* gene expression blockade by using a specific HER-2 siRNA sequence was able to partially restore rapamycin-driven cyclin D3 down-regulation as well (Fig. 6E), which indicates that the rapamycin effect on cyclin D3 is dependent on HER-2 expression and activity. It has been suggested a link between HER-2 overexpression and deficient expression



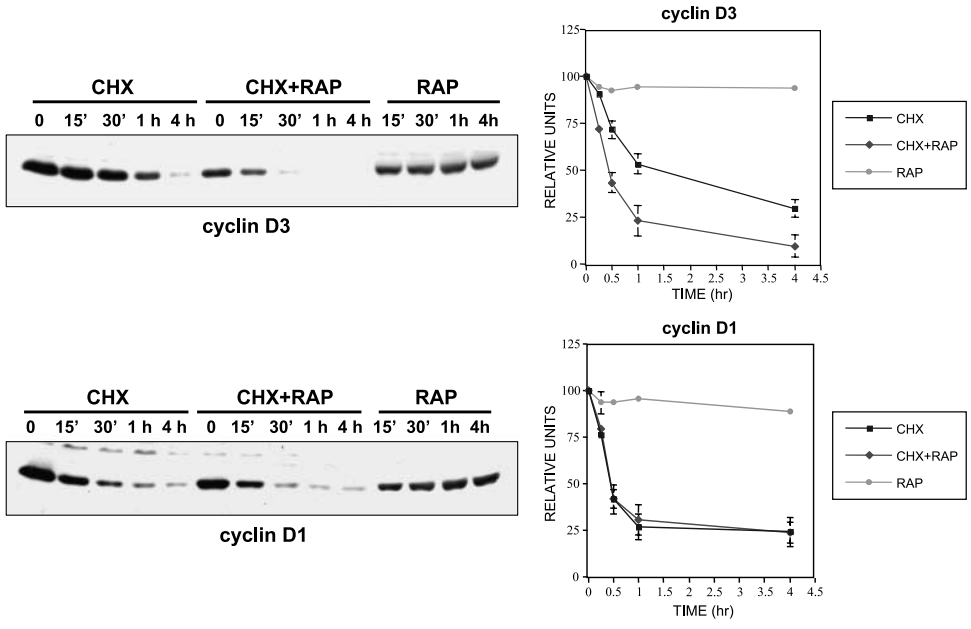


**Figure 5.** Effect of rapamycin on D-cyclins protein turnover. SKBr-3 cells were pretreated with rapamycin (10 nmol/L) for 16 h or left untreated, and cycloheximide (10 μg/mL; *CHX*) was added in the presence or absence of rapamycin for the times indicated. Cyclin D1 and cyclin D3 protein levels were analyzed by Western blot, and bands were quantified and represented as the average of four separate experiments.

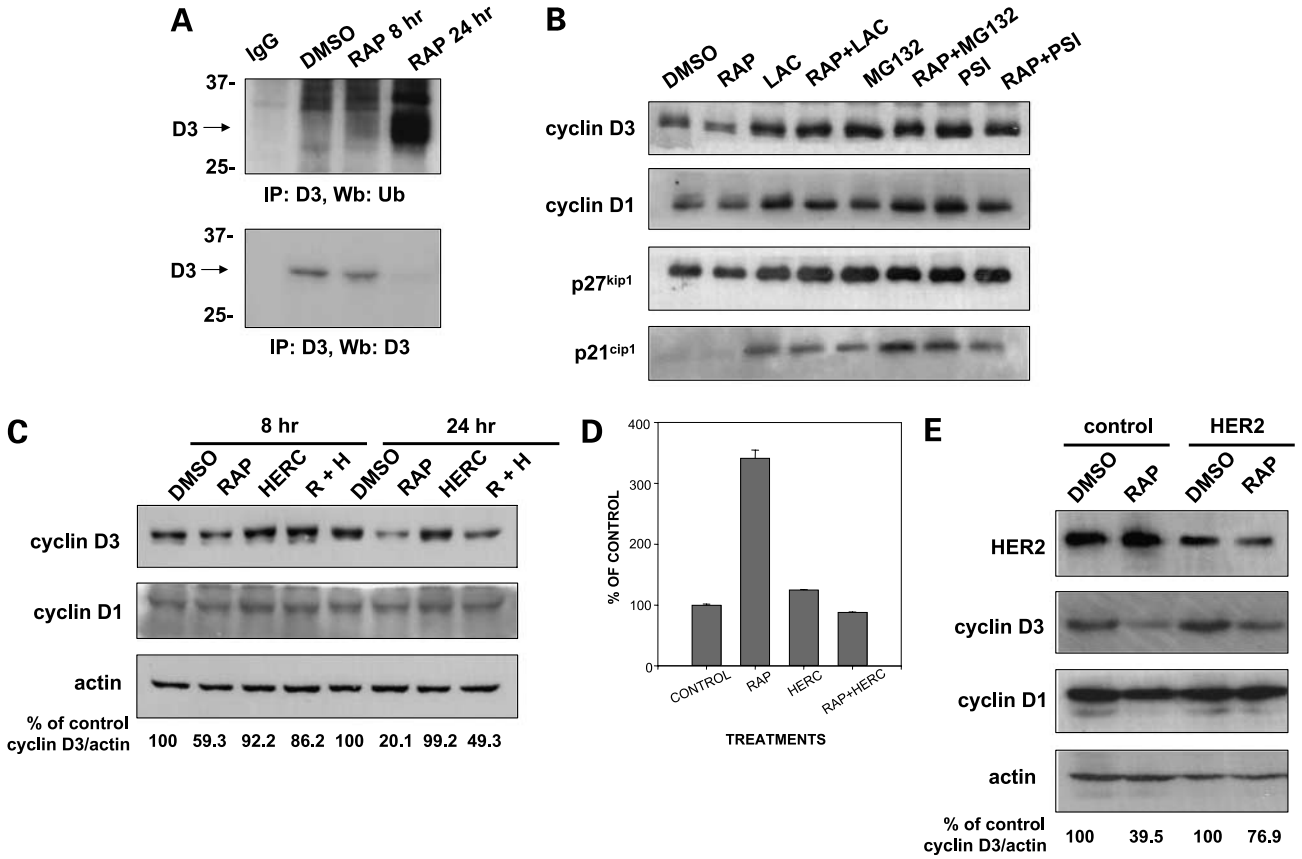


**Figure 6.** Ubiquitination of cyclin D3 protein. **A,** SKBr-3 cells were treated with DMSO or 10 nmol/L rapamycin for 8 and 24 h. Cell extracts were immunoprecipitated with cyclin D3 antibody and probed against ubiquitin to detect ubiquitinated forms and against cyclin D3 as a loading control. Effect of proteasome inhibitors. **B,** SKBr-3 cells were nontreated or treated with proteasome inhibitors (10 μmol/L lactacystin (*LAC*), 10 μmol/L MG132, and 25 μmol/L proteasome inhibitor I (*PSI*) 30 min before the addition of 10 nmol/L rapamycin for 8 h. The levels of cyclin D3, cyclin D1, $p27^{kip1}$, and $p21^{cip1}$ were analyzed by Western blot. Effect of Herceptin on D-cyclins. **C,** SKBr-3 cells were pretreated with 20 μg/mL Herceptin (*HERC*) 30 min before addition of 10 nmol/L rapamycin and incubated for 8 or 24 h. Cyclin D1, cyclin D3, and actin protein levels were analyzed by Western blot. Protein bands were quantified and cyclin D3 protein bands were normalized to actin protein bands and referred as percentage of control. Effect of Herceptin on proteasomal activity. **D,** SKBr-3 cells were pretreated with 20 μg/mL Herceptin before addition of DMSO or 10 nmol/L rapamycin for 24 h and the activity of the proteasome was determined as described in Material and Methods and represented as the average of three experiments. **E,** inhibition of *HER-2* gene expression. SKBr-3 cells were transfected with HER-2 siRNA or control siRNA before treatment with DMSO or 10 nmol/L rapamycin for 16 h. HER-2, cyclin D3, cyclin D1, and actin protein levels were analyzed by Western blot. Protein bands were quantified and cyclin D3 protein bands were normalized to actin protein bands and referred as percentage of control.

of components of the antigen-processing pathway, including proteasome activators (35). However, the relationship between HER-2 overexpression and activation and regulation of the proteasome-ubiquitin pathway needs to be investigated. Cyclin D1 and cyclin D3 seem to be regulated in a different manner in HER-2-overexpressing cells. It has been shown that protein stability is an important component of regulation of D-cyclins (36, 37), which are essential regulators of the $G_1$ phase of the cell cycle and are rate limiting in the $G_1$-S phase transition. Here, we show that rapamycin down-regulates cyclin D3 specifically but not cyclin D1. D-cyclin isoforms have tissue and cell specificity and may play a different role during normal and tumor development. As a matter of fact, a differential role of cyclin D3 in cancer progression has been suggested (38, 39). We have shown in this report that HER-2-overexpressing breast cancer cells are responsive to rapamycin. Their growth inhibition is due to a $G_1$ arrest that may be initially caused by cyclin D3 down-regulation. Rapamycin seems to destabilize cyclin D3 protein that may be targeted to degradation by the ubiquitin-proteasome pathway at a faster rate. Rapamycin also causes a decrease of D-cyclin-associated kinase activities and a subsequent decrease of cdk2 activity, all these factors contributing to the $G_1$ arrest observed in this system. The effects seen in these cells are not observed in other cancer cells that do not overexpress HER-2, suggesting a possible link between HER-2 overexpression and cyclin D3 regulation through the mTOR signaling pathway that needs to be further investigated.

We have shown in this report that cells that overexpress HER-2 and have a deranged PI3K/Akt/mTOR pathway are susceptible to growth inhibition and cell cycle arrest by rapamycin. This suggests that mTOR inhibitors, such as

rapamycin and its derivatives, CCI-779, RAD001, or AP23573, are potential therapeutic agents that can be used for the treatment of patients that present a deranged PI3K/Akt pathway, such as HER-2-overexpressing tumors. Indeed, rapamycin is a potent inhibitor of tumor growth in transgenic mice bearing an activated HER-2 form (40). The fact that rapamycin is cytostatic makes it a good candidate to be used in combination with other antitumor agents (41), as it can sensitize tumors that are resistant to other chemotherapeutic agents (42, 43). The relevance of targeted therapies is very evident nowadays and the potential role of rapamycin and its derivatives is becoming more important for the treatment of breast cancer patients (44, 45). We have shown in this report that rapamycin-based therapies would be very useful in a subset of breast cancer patients that overexpress HER-2 and are resistant to the conventional and more general chemotherapeutic agents that are being used in the clinic.

**Acknowledgments**

We thank José A. Ferragut for critical reading of the article and María Romero for technical support.

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