Inhibitors of mTOR Reverse Doxorubicin Resistance Conferred by PTEN Status in Prostate Cancer Cells[1]

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ABSTRACT

Phosphatase and tensin homologue deleted from chromosome 10 (PTEN) is a lipid phosphatase with putative tumor suppressing abilities, which is frequently mutated in prostate cancer. Loss of PTEN leads to constitutive activation of the phosphatidylinositol 3′-kinase/serine-threonine kinase (Akt) signal transduction pathway and has been associated with resistance to chemotherapy. This study aimed to determine the effects of PTEN status and treatment with rapamycin, an inhibitor of mTOR, in the response of prostate cancer cell lines to doxorubicin. The DU-145 PTEN-positive cell line was significantly more susceptible to the antiproliferative effects of doxorubicin as compared with the PTEN-negative PC-3 cell line. Transfection of PTEN into the PC3 cells decreased the activation of Akt and the downstream mTOR-regulated 70-kDa S6 (p70^{s6k}) kinase and reversed the resistance to doxorubicin in these cells, indicating that changes in PTEN status/Akt activation modulate the cellular response to doxorubicin. Treatment of PC-3 PTEN-negative cells with rapamycin inhibited 70-kDa S6 kinase and increased the proliferative response of these cells to doxorubicin, so that it was comparable with the responses of PTEN-positive DU-145 cells and the PC-3-transfected cells. Furthermore, treatment of mice bearing the PTEN-negative PC-3 prostate cancer xenografts with CCI-779, an ester of rapamycin in clinical development combined with doxorubicin, inhibited the growth of the doxorubicin-resistant PC-3 tumors confirming the observations *in vitro*. Thus, rapamycin and CCI-779, by interacting with downstream intermediates in the phosphatidylinositol 3′-kinase/Akt signaling pathway, reverse the resistance to doxorubicin conferred by PTEN mutation/Akt activation. These results provide the rationale to explore in clinical trials whether these agents increase the response to chemotherapy of patients with PTEN-negative/Akt active cancers.

INTRODUCTION

The *PTEN*[3] gene (also know as *MMAC1* or *TEP1*), is located in *chromosome 10* and codifies a 403-amino acid dual-specific phosphatase with putative tumor suppressing abilities (1, 2). Inactivating mutations in the *PTEN gene* have been related to the processes of tumor development as progression from several perspectives. Inherited mutations of *PTEN* are believed to cause the Cowden's and Bannayan-Zonana inherited cancer syndromes that predispose to the development of multiple tumors (3). Acquired mutations in the *PTEN gene* have been reported in several tumor types, including up to 30–60% of prostate cancer tumors, and are associated with advanced

stage, aggressive clinical course, and poor prognosis (4–8). Furthermore, *PTEN* +/− heterozygous mice develop spontaneous tumors in multiple locations and, when mated with *p27KIP1*$^{−/−}$ mice, develop spontaneous prostate cancer with a full penetrance by the age of 3 months (9). Although the PTEN protein has been implicated in multiple important cellular functions pertaining to cell cycle regulation and survival, its best characterized function relates to the regulation of the of the PI3k/Akt signaling pathway (10, 11). PTEN cleaves the D3 phosphate of PIP3, an important activator of Akt, and, therefore, mutations of *PTEN* result in constitutive activation of the PI3k/Akt signaling pathway (12). Embryonic and tumor cells from *PTEN* +/− mice have increased activation of Akt, which is associated with activation of the transcriptional regulator p70^{s6k}, a downstream intermediate in the PI3k/Akt signaling pathway (13). Activation of the PI3k/Akt signal transduction pathway has been related to many important oncological processes such as cell proliferation and survival (14, 15). Importantly, *PTEN* mutations/deletions and activation of Akt have been associated with resistance to chemotherapy and hormone therapy in preclinical cancer models (16). Restoration of the PTEN function by adenoviral gene transfer corrects the cellular anomalies conferred by *PTEN* mutations and has antiproliferative effects (17).

Our group has been interested in the development of specific pharmacological treatments for patients with PTEN negative tumors. Considering the important function of PI3k/Akt activation in situations of PTEN deficiency, our efforts focused on the exploration of pharmacological modulators of this signal transduction pathway. In initial studies, we observed that breast cancer cells with defective *PTEN* gene became susceptible to the antiproliferative effects of the mTOR inhibitor rapamycin (18). mTOR is a serine-threonine kinase that is activated by Akt and regulates the function of the translational regulators p70^{s6k} and 4EB-P1 (reviewed in Ref. 19). In preclinical studies, rapamycin resulted in G$_1$ cell cycle arrest, induced apoptosis, and had antiproliferative effects in several cancer models. An ester of rapamycin, CCI-779, is currently being developed for the treatment of patients with cancer. Recent data indicate that CCI-779 inhibits the activation p70^{s6k} in *PTEN*-defective tumor models and has preferential antiproliferative and preventive actions in tumor models with this genetic defect (13, 20, 21).

In this study, we determined the effects of rapamycin on the chemosensitivity of prostate cancer cell lines with variant PTEN expression. We found a correlation between PTEN negativity and resistance to chemotherapy. Introduction of the *PTEN* gene into PTEN-negative cells reversed the resistance to doxorubicin, outlining the importance of the *PTEN* gene in chemosensitivity. Importantly, combined treatment with doxorubicin and rapamycin had similar effects to PTEN transfection in doxorubicin chemosensitivity in PTEN-negative cells. These results indicate that rapamycin is capable of restoring the susceptibility of PTEN negative cell lines to chemotherapy and represents the rational to explore this hypothesis in the clinic.

MATERIALS AND METHODS

Cell Lines. PC-3 and DU-145 prostate cancer cells were obtained from the American Type Culture Collection and maintained in improved minimum essential medium (MEM) Zn^{2+} option (Life Technologies, Inc., Gaithersburg,

MD) supplemented with 10% FBS (Sigma, St. Louis, MO) and 6 ng/ml of bovine insulin (Sigma).

Western Blot Analysis. Cells were serum starved for 18 h, stimulated with 10% FBS for the indicated times and subsequently harvested in SDS lysis buffer [62.5 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 10 mM ortho-sodium-vanadate, 3 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml antipain], and 20 μg of protein extract were resolved on a polyacrylamide gel and then transferred to nitrocellulose membranes. Immunoblotting was done using antibodies against PTEN (Cascade, Winchester, MA), phosphorylated and total Akt (Cell Signaling Technology, Beverly, MA), phosphorylated and total p70^{s6k} (Cell Signaling Technology), and actin (Santa Cruz Biotechnology, Santa Cruz, CA). Protein was detected by chemiluminescence (Amersham, Arlington Heights, IL). Densitometry analysis employed NIH Image 1.62 software.

Cell Viability Assay. Cell viability was assessed by MTT (Sigma) dye conversion at 570 nm following manufacture's instructions. Briefly, cells were seeded 5×10^3/well in a 96-well flat-bottomed plate. Cells were allowed to grow 24 h, then placed in serum-starved conditions for 18 h. Cells were then treated with increasing concentrations of rapamycin (Sigma) and/or doxorubicin in the presence of 10% FBS. After 96 hr, 20 μl of MTT (5 mg/ml in PBS) were added to each well. After 3 h of incubation at 37°C, cells were lysed by the addition of 0.1 N HCl in isopropanol. Response to drug treatment was assessed by standardizing treatment groups to untreated control.

Expression Vectors and Constructs. The expression plasmid for PTEN, *CMV-PTEN*, has been described previously (22).

Generation, Selection, and Analysis of Stable Transfectants. PC-3 cells were transfected with the CMV-PTEN construct (PC-3PTEN) or with empty plasmid as control (PC-3pcDNA3.1+) using FuGene 6 (Boehringer Mannheim, Indianapolis, IN). One day after transfection, cells were placed into the selection medium containing 0.4 mg/ml G418 (Life Technologies, Inc.). Fourteen days after selection, individual G418-resistant colonies were subcloned. Protein expression was analyzed by Western blot using the PTEN antibody described above.

Reverse Transcription-PCR. Total RNA from PC-3, DU-145, PC-3PTEN, and PC-3pcDNA3.1+ cells was isolated using Trizol (Life Technologies, Inc.), following manufacture's instructions. Total RNA (2 μg) was reversed transcribed using Superscript II (Life Technologies, Inc.), following company instructions. cDNA was amplified by PCR using Platinum Taq DNA polymerase (Life Technologies, Inc.) consisting of 30 cycles with specific primers (sense: 5′-ACAGCCATCATCAAAGAGATCG-3′ and antisense: 5′-CACCACACACAGGTAACGGC-3′) at 94°C for denaturing, 54°C for annealing, and 72°C for extension. As an internal control, amplification of the RPS3 mRNA was carried out by reverse transcription-PCR using specific primers (sense: 5′-GTTCATCATGGAGAGTGG-3′ and antisense: 5′-GCAT-GATGATCTTCACCTTGA-3′). PCR products were analyzed by 2% agarose gel electrophoresis in the presence of ethidium bromide for UV light transilluminator visualization.

Data Analysis. Experiments have been repeated five times, and the average and 95% confidence interval were determined.

Animal Study. All animal studies were conducted according to guidelines of the American Association of Laboratory Animal Care. Four to 6-week-old athymic mice were inoculated with 1×10^6 PTEN-negative PC-3 cells at each flank in PBS containing 20% Matrigel. Treatment was initiated when tumors reached ~200 mm³. Tumor size was measured every other day with a caliper and tumor volume was calculated by following formula: [length × width²]/2. CCI-779 (Wyeth Research, Collegeville, PA) was initially dissolved in ethanol at a concentration of 50 mg/ml and then diluted to 2 mg/ml in 0.15 M NaCl, 5% Tween 20, and 5% polyethylenglycol 400. Mice were treated with 10 mg/kg i.p. on days 1–5 with fresh drug solutions every day. Doxorubicin (Adria Labs, Columbus, OH) was administered 10 mg/kg i.v. on day 1 only. Control mice received i.p. injections with vehicle only. Growth inhibition was calculated by tumor volume of treated mice divided by volume of control mice (*T/C*).

RESULTS AND DISCUSSION

PC-3 and DU-145 Have Distinct PTEN Expression and Akt Activation. Loss of *PTEN* is associated with a hyperactivated PI3k/Akt pathway. To assess the role of PTEN status in response to drug treatment, PC-3 and DU-145 prostate cancer cells were first screened by Western blot analysis to determine the status of PTEN expression and phosphorylation of Akt, its main molecular target and p70^{s6k}, an mTOR-regulated downstream intermediate in the PI3k/Akt signaling pathway. In Fig. 1*A*, PC-3 cells demonstrate no PTEN expression, which correlated to high levels of phosphorylated Akt and phosphorylated p70^{s6k}. In contrast, the DU-145 cells displayed high PTEN levels, which correlated with no detectable levels of phosphorylated Akt and low levels of p70^{s6k}. We next tested whether prostate cancer cells with variant PTEN status responded differently to the cytotoxic effects of doxorubicin, an inhibitor of topoisomerase II (Fig. 1*B*). PTEN-negative PC-3 and PTEN-positive DU-145 cells were treated with increasing concentrations of doxorubicin (1 ng/ml, 10 ng/ml, 50 ng/ml). Cytotoxic response was measured by MTT assay, and the surviving cell fraction was calculated standardized to untreated controls. The PTEN-negative PC-3 prostate cancer cells were more resistant to the antiproliferative effects of doxorubicin as shown in Fig. 1*B*. This finding is in agreement with published reports indicating that Akt hyperactivation confers resistance to chemotherapy (16). Considering the high frequency of *PTEN* defects in prostate cancer and the fact that topoisomerase II inhibitors are widely use to treat hormone refractory prostate cancer, this finding has important clinical consequences.

PTEN Status Predicts Response to Treatment with Rapamycin and Doxorubicin in PTEN-transfected PC-3 Cells. To further evaluate the effect of PTEN in prostate cancer cells in response to treatment with signal transduction inhibitors and cytotoxic therapy, we stably transfected PTEN-negative PC-3 cells with a PTEN expres-
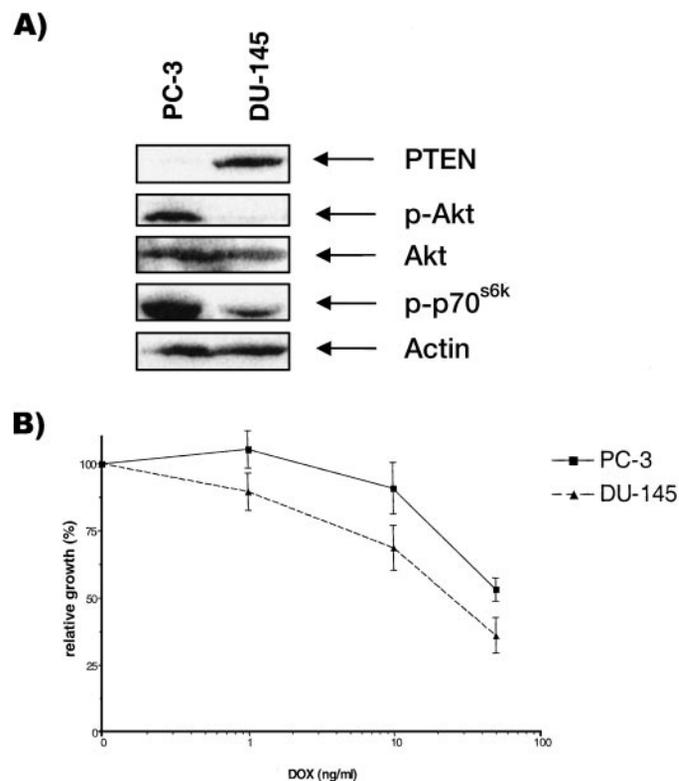

Fig. 1. PTEN status confers chemoresistance in prostate cancer cells. *A*, cells were grown in culture media containing 10% FBS, were serum starved for 24 h, and lysed in SDS lysis buffer. Twenty μg of protein lysate were separated on 10% SDS-PAGE by electrophoresis and immunoblotted for PTEN, phosphorylated Akt, total Akt, p-p70^{s6k}, and actin. PC-3 prostate cancer cells displayed loss of PTEN, associated with constitutive-activated Akt, whereas DU-145 showed PTEN expression and no activated Akt. *B*, treatment with increasing doses of doxorubicin (1 ng/ml, 10 ng/ml, 50 ng/ml) showed higher cytotoxicity in the PTEN-positive DU-145 cells when compared with the PTEN-negative PC-3 cells.

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sion plasmid. After stable transfection, reverse transcription-PCR analysis identified PTEN mRNA in PTEN-transfected PC-3 cells, and no PTEN mRNA in the parental PC-3 cells. PTEN-positive DU-145 cells were used as control (Fig. 2*A*). Transfected cells were characterized for PTEN mRNA expression and levels of activated Akt by reverse transcriptase-PCR and Western blot analysis, respectively. Under serum-starved conditions, densitometry analyses of three independent experiments showed a 50% decrease in activated Akt in the PTEN-transfected PC-3 cells, which correlated with expression levels of PTEN (analyses not shown). However, after stimulation with 10% FBS, equal levels of activated Akt were observed (Fig. 2*B*). To further assess the response to doxorubicin in PTEN-deficient prostate cancer cells, we treated the PTEN-transfected PC-3, parental PC-3, and PTEN-positive DU-145 cells with increasing concentrations of doxorubicin (1 ng/ml, 10 ng/ml, 50 ng/ml). The PTEN-transfected PC-3 cells showed higher responses to the cytotoxic agent doxorubicin that was similar to the response observed in the DU-145 cells (Fig. 3*A*). No differences, however, were observed with regard to response to the mTOR inhibitor rapamycin. Although rapamycin and analogue agents have been demonstrated to have preferential antitumor effects in PTEN-negative models with >10-fold differences in IC_{50} concentration, the IC_{50} against the DU-145 and PC-3 prostate cancer cells is only 2-fold (21). This finding may be explained by the presence of an additional alteration in the DU-145 cells such as constitutive activation of mTOR, which makes them susceptible to rapamycin. In addition, no differences were observed in PC-3 parental *versus* PTEN-transfected cells with regard to response to rapamycin (Fig. 3, *B* and *C*). However, under conditions of serum stimulation, the PTEN-transfected cells still activate Akt and p70^{s6k}, although to a lesser degree than the parental cells (Fig. 4*C* for p70^{s6k} activation). Therefore, it appears that the relationship between Akt activation and

Fig. 3. Stable PTEN expression leads to doxorubicin susceptibility but not to increased response to rapamycin. *A*, treatment with increasing concentrations of doxorubicin (1 ng/ml, 10 ng/ml, 50 ng/ml) displayed higher susceptibility for the PTEN-expressing cells (DU-145, PC-3PTEN). At the 10 ng/ml dose level, PTEN-transfected PC-3 and DU-145 cells showed an increase in cytotoxic response by 24 and 25%, respectively. *B*, parental PC-3 and PTEN-transfected PC-3 cells were treated with increasing concentrations of rapamycin (10 nM, 20 nM, 50 nM). Both cell lines displayed similar drug response. *C*, PTEN-positive DU-145 and PTEN-negative PC-3 cells were treated with increasing concentrations of rapamycin (10 nM, 20 nM, 50 nM) and revealed equal sensitivity to rapamycin. Drug response was detected by MTT assay as described above.
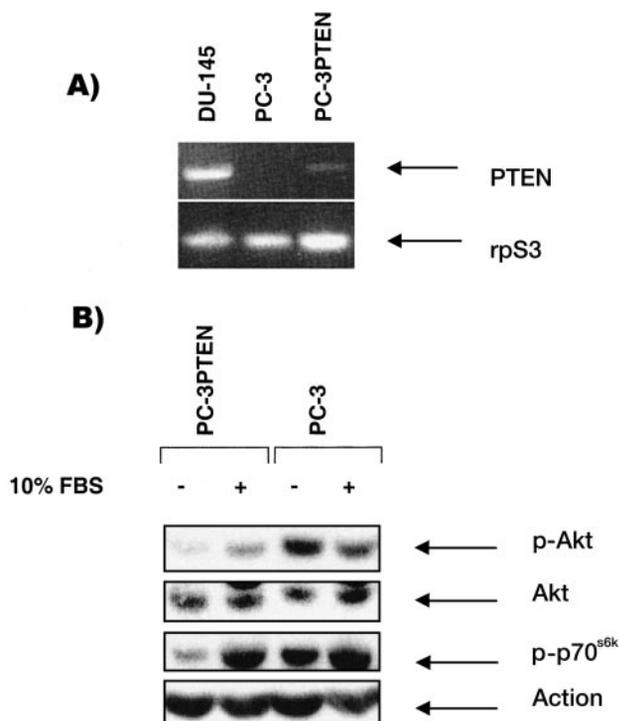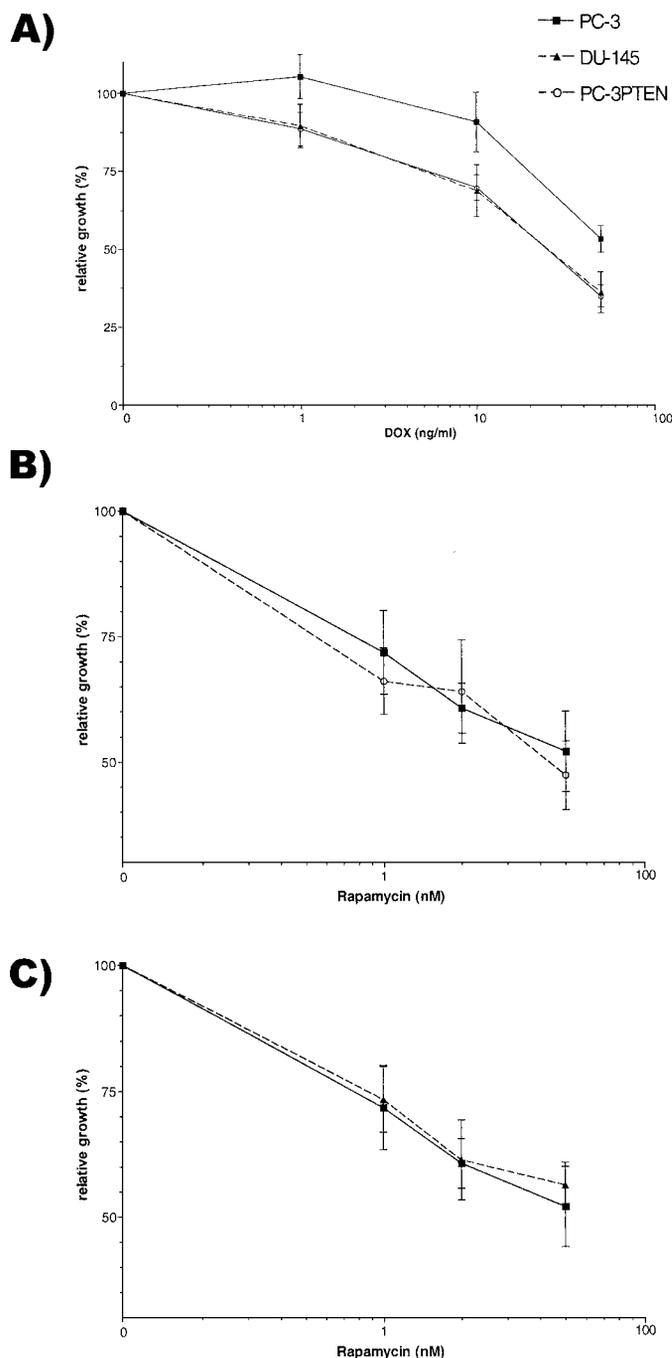

Fig. 2. Transfection of PTEN leads to loss of hyperactivated Akt. *A*, reverse transcription-PCR demonstrated PTEN mRNA expression in DU-145 and PC-3 transfectant cells but not in PC-3 parental cells. Ribosomal S3 was used as an internal control rpS3. *B*, parental PC-3 and PTEN-transfected PC-3 cells were serum starved for 24 h, then stimulated as indicated with 10% FBS. Expression of PTEN, phosphorylated Akt, phosphorylated p70^{s6k}, and actin were determined by Western blot analysis.

response to doxorubicin and rapamycin is probably complex and that although minor differences in Akt activation are sufficient to confer resistance to doxorubicin, larger differences are needed to confer differential susceptibility to rapamycin. Studies are in progress to determine the precise effects of doxorubicin and rapamycin in induction of apoptosis and cell cycle arrest in the context of differential Akt activation.
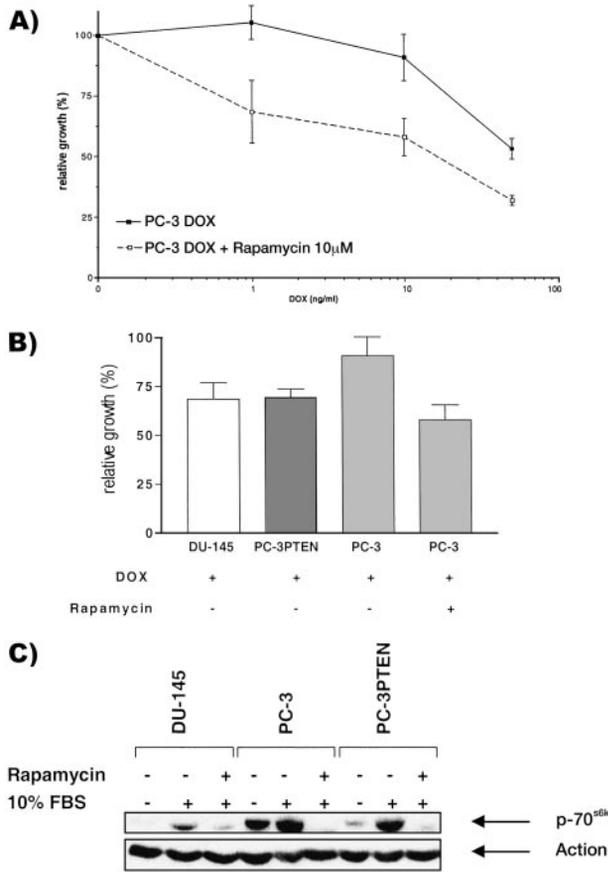
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Fig. 4. Rapamycin reverses drug resistance conferred by loss of PTEN. *A*, cotreatment with 10 nM rapamycin in doxorubicin (DOX) (1 ng/ml, 10 ng/ml, 50 ng/ml)-treated PC-3 cells showed a marked increase in drug response. *B*, PTEN-positive DU-145, PTEN-transfected PC-3, and parental PC-3 cells were treated with 10 ng/ml doxorubicin. Drug response was assessed by MTT assay. In addition, PTEN-negative PC-3 cells were cotreated with 10 nM rapamycin and showed similar doxorubicin susceptibility as PTEN-positive cells. *C*, PTEN-positive prostate cancer cells (DU-145, PC-3PTEN) showed lower basal activity of p70^{s6k} but remain inducible upon stimulation with 10% FBS. Treatment with 10 nM rapamycin inhibited the activation of p70^{s6k} by 10% FBS.

Rapamycin Reverses the Resistance to Doxorubicin in PTEN-negative Cells. Subsequently, we explored whether treatment with rapamycin had similar effects to PTEN replacement with regard to doxorubicin treatment. Rapamycin treatment inhibited p70^{s6k} phos-

phorylation in all treated cell lines as shown in Fig. 4*C*. Cotreatment of PTEN-negative PC-3 cells with doxorubicin in combination with rapamycin increased the susceptibility of these cells to doxorubicin (Fig. 4*A*) to the levels observed in the PTEN-positive DU-145 and PC-3-transfected cells (Fig. 4*B*). Subsequently, we tested this hypothesis in a nude mouse model using the PTEN-negative PC-3 prostate cancer xenografts (Fig. 5). Treatment with CCI-779 in combination with doxorubicin decreased the *T/C* ratio from 69% with doxorubicin alone to 29% with the combination by day 33. Whereas, CCI-779 as a single agent resulted in a *T/C* ratio of 40%.

The results of these studies confirm previous observations linking defective PTEN and activated Akt with resistance to chemotherapy. Furthermore, transfection of the PTEN gene decreases Akt activation and restores the susceptibility of the cells to doxorubicin, indicating that PTEN function is critical in the response of these cells to chemotherapy. The mechanism mediating the protection of Akt from doxorubicin cytotoxic effects is, however, unknown. Induction of cell cycle arrest or inhibition of apoptosis or a combination of both could be responsible for resistance to doxorubicin. Recently, it has been demonstrated that defects in the *PTEN* gene are associated with resistance to chemotherapy in prostate cancer cells because of bcl-2 expression and inhibition of apoptosis, suggesting that protection from apoptosis could be one of the mechanisms involved in this process (23). Treatment with rapamycin inhibited p70^{s6k} phosphorylation in the PTEN-negative cell lines, reversing their resistance to doxorubicin in the same magnitude as transfection of the *PTEN* gene. Although the principal role of p70^{s6k} is to control the translation of G$_1$ phase mRNAs, recent studies indicate that p70^{s6k} is also involved in the regulation of apoptosis through phosphorylation and inactivation of the proapoptotic protein BAD (24). Additional studies are in progress to precisely define the mechanisms by which rapamycin reverses PTEN-mediated resistance to chemotherapy.

In summary, these data indicate that PTEN negativity is associated with resistance to chemotherapy in prostate cancer cells. Considering the high frequency of PTEN mutations resulting in Akt activation in prostate cancer cells, this finding has significant therapeutic implications. Importantly, cotreatment with the mTOR inhibitor rapamycin restored the response to doxorubicin in PTEN-negative prostate cancer cells in culture. Also, in nude mice with PTEN-negative prostate tumor xenografts, CCI-779, an ester of rapamycin, effectively inhibited tumors growth and restored the response to doxorubicin, supporting the idea that combined treatment regimens with both agents will

Fig. 5. CCI-779 enhances doxorubicin response in PTEN-negative prostate xenografts. Male nude mice were inoculated with PTEN-negative PC-3 cells on both flanks. Mice were stratified according to tumor volume upon an approximate size of 200 mm^3 into four groups (day 0). Treatment was initiated the next day and consisted of vehicle i.p. days 1–5 (◆), doxorubicin 10 mg/kg i.v. day 1 (◇), CCI-779 10 mg/kg i.p. days 1–5 (▲), or a combination of both treatments at the same doses and schedules used in the single agent administrations (△). Doxorubicin (DOX) imperceptibly delayed tumor growth, whereas CCI-779 induced growth inhibition as a single agent and additive antitumor effects in the combined treatment arm. *Arrows* mark injection dates.

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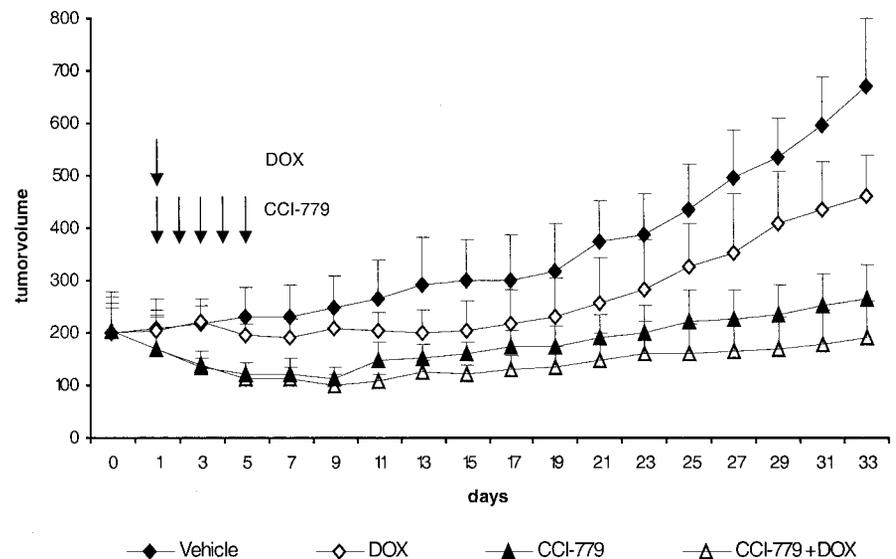
be beneficial. CCI-779 currently is in clinical development in patients with several tumor types. These data provide the rationale to conduct studies of CCI-779 in combination with chemotherapy in patients with PTEN-defective cancers.

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