Cancer Therapy: Preclinical

Concurrent and Sequential Administration of Chemotherapy and the Mammalian Target of Rapamycin Inhibitor Temsirolimus in Human Cancer Cells and Xenografts

Andrea S. Fung, Licun Wu, and Ian F. Tannock

Abstract

Purpose: Optimal scheduling of cycle-active chemotherapy with (initially cytostatic) molecular-targeted agents is important to maximize clinical benefit. Concurrent scheduling might allow up-regulation of cell death pathways at the time of chemotherapy, whereas sequential treatments might maximize inhibition of repopulation and avoid putting tumor cells out of cycle when administering cycle-active chemotherapy. We compared the effects of concurrent and sequential administration of chemotherapy and the mammalian target of rapamycin (mTOR) inhibitor temsirolimus (CCI-779) on tumor cells and xenografts.

Experimental Design: Human prostate cancer PC-3 and LnCaP, and human breast cancer MDA-468 cells and xenografts were treated with chemotherapy (docetaxel and 5-fluorouracil, respectively) and temsirolimus, using concurrent and sequential treatment schedules. Cell killing and repopulation were evaluated by clonogenic assays. Cell cycle analysis was done using flow cytometry. Effects on xenografts were assessed by tumor growth delay.

Results: The proliferation of all cell lines was inhibited by temsirolimus in a dose-dependent manner; PTEN negative PC-3 and mutant LnCaP cells were more sensitive than PTEN-negative MDA-468 cells. Temsirolimus inhibited cell cycle progression from G_1 to S phase in all cell lines. Combined treatment had greater effects than temsirolimus or chemotherapy alone: for PC-3 and LnCaP xenografts, concurrent treatment seemed superior to sequential scheduling, whereas MDA-468 cells and xenograft tumors did not show schedule dependence.

Conclusions: Combined treatment with temsirolimus and chemotherapy had a greater therapeutic effect than monotherapy; concurrent scheduling was more effective for PC-3 and LnCaP cells and xenografts that were sensitive to temsirolimus. (Clin Cancer Res 2009;15(17):5389–95)

Clinical benefit from chemotherapy is limited by systemic toxicity and by drug resistance. Most studies of drug resistance have concentrated on cellular and molecular mechanisms operating at the level of a single cell (reviewed in ref. 1), but limited drug distribution within tumors and repopulation of surviving tumor cells between cycles of chemotherapy are important and neglected causes of clinical drug resistance (2–5). Repopulation of tumor cells between successive courses of chemotherapy may accelerate, and may lead to acquired resistance in the absence of changes in the intrinsic sensitivity of the tumor cells (2, 3, 6). Many molecular-targeted agents are being introduced in the clinic; their initial effects are usually cytostatic, and these agents have considerable potential for inhibiting repopulation. For example, cetuximab, an inhibitor of the epidermal growth factor receptor, has been shown to increase survival of patients receiving radiotherapy for head and neck cancer (7), and the most likely mechanism is inhibition of repopulation during the course of radiotherapy. Inhibition of repopulation by cytostatic agents between cycles of chemotherapy is more complex because most chemotherapy drugs are more active against cycling cells, and the outcome may depend markedly on schedule. Concurrent scheduling might allow up-regulation of cell death pathways at the time of chemotherapy, whereas sequential treatments might maximize inhibition of repopulation and avoid putting tumor cells out of cycle when administering cycle-active chemotherapy.

Temsirolimus (CCI-779), an inhibitor of the mammalian target of rapamycin (mTOR), is a molecular-targeted agent that

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Translational Relevance

Temsirolimus, an inhibitor of the mammalian target of rapamycin, was approved recently by the Food and Drug Administration for the treatment of renal cancer. Limited studies have investigated the effects of temsirolimus in combination with chemotherapy. Our study investigates the effects of temsirolimus and chemotherapy on prostate and breast cancer cells and xenografts, with emphasis on scheduling (sequential or concurrent) of combined therapy and possible effects of temsirolimus to inhibit repopulation between cycles of chemotherapy. We have shown that temsirolimus administered concomitantly with docetaxel is more effective in delaying regrowth of prostate PC-3 and LnCaP xenografts in nude mice when compared with either agent alone. Further studies should determine optimal dosing and scheduling in the clinic, but our studies suggest that combined treatment with temsirolimus and docetaxel might be beneficial in the treatment of prostate cancer.

has shown considerable activity in preclinical and clinical studies (8–15). The mTOR pathways are important in promoting cell proliferation and cell survival, and temsirolimus has marked effects to inhibit cell proliferation. The product of the *PTEN* tumor suppressor gene is a phosphatase that down-regulates the phosphoinositide 3-kinase/Akt pathway. Loss of PTEN is correlated with up-regulated mTOR activity and can render tumors particularly sensitive to mTOR inhibitors (9). Rapamycin and its analogue temsirolimus down-regulate translation of specific mRNAs required for cell cycle progression from G_1 to S phase (8, 9). Temsirolimus has shown antiproliferative activity against a wide range of cancers in preclinical models and clinical trials and is now approved by the Food and Drug Administration for the treatment of renal cancer (11, 13, 15). Tumors with PTEN mutations are particularly sensitive to temsirolimus; for example, our previous data indicated that PTEN-negative human prostate cancer PC-3 cells and xenografts are quite sensitive to temsirolimus (14).

There are few clinical studies that have addressed sequencing of cytotoxic chemotherapy with molecular-targeted agents. Tamoxifen has been used concurrently or sequentially after chemotherapy, in trials of adjuvant therapy for breast cancer. The randomized Intergroup Trial 0100 showed that sequential treatment was more beneficial than concurrent administration for postmenopausal women with node-positive, estrogen receptor or progesterone receptor-positive disease (16, 17), and a second trial reported similar trends (18). Preclinical studies also suggest that inhibitors of the epidermal growth factor receptor tyrosine kinase such as gefitinib lead to better outcome when administered sequentially with chemotherapy, compared with concurrent treatment (19, 20). We were unable to identify studies that have investigated concurrent or sequential scheduling of chemotherapy and temsirolimus.

In the present study, we evaluate the effects of concurrent and sequential administration of chemotherapy and the mTOR inhibitor temsirolimus on human prostate and breast cancer cells and xenografts.

Materials and Methods

Cell lines and mice. Human prostate cancer PC-3 cells and LnCaP cells were maintained in Ham's F-12K medium supplemented with 2 mmol/L L-glutamine and RPMI, respectively, and human breast cancer MDA-468 cells were cultured in α-MEM. All media contained 10% fetal bovine serum, 1% penicillin, and streptomycin. All cell lines were purchased from the American Type Culture Collection.

Athymic nude mice (ages 4-6 wk old) were purchased from the Harlan Sprague-Dawley laboratory animal center and acclimatized in the animal colony for 1 wk before experimentation. The animals were housed in microisolator cages, five per cage, in a 12-h light/dark cycle. The animals received filter-sterilized water and sterile rodent food *ad libitum.*

Drugs and preparation. The mTOR inhibitor temsirolimus was obtained from Wyeth-Ayerst Laboratories; it was stored as a dry powder at 4°C and suspended in 100% ethanol on the day of use. A stock solution of temsirolimus was diluted to a concentration of 2 mmol/L using 5% Tween 80 (Sigma) and 5% polyethylene glycol 400 (Sigma). Docetaxel (Aventis Pharmaceuticals, Inc.) and 5-fluorouracil (5-FU; Mayne Pharma, Inc.) were obtained from the hospital pharmacy.

Effects of temsirolimus and chemotherapy on cell proliferation in vitro. PC-3 and LnCaP cells (10^6) were plated into multiple 25-cm² flasks and treated with various doses of temsirolimus (0, 100, 500, and 1,000 nmol/L) for 3 d; or docetaxel (0, 5, 10, 50, and 100 nmol/L) for 24 h, and then washed with PBS thrice. MDA-468 cells were evaluated in similar experiments using 5-FU (10 μmol/L) for 24 h. Cells were counted using a Coulter Counter to determine the effect of the above treatment on cell growth. Colony-forming assays were done to evaluate the surviving fraction.

Concurrent or sequential treatment of cultured cells. Optimal doses of temsirolimus and docetaxel or 5-FU selected from the above experiments were used in studies evaluating combined treatment. PC-3 and LnCaP cells (10^6) were plated and allowed to grow for 1 d. Temsirolimus was added concurrently with or sequentially after docetaxel (Fig. 1A). For concurrent treatment, both drugs were added for 24 h, followed by three washes with PBS, and temsirolimus was added for a further 2 d and then washed out. For sequential treatment, docetaxel was added for 24 h, followed by washing, and after a further 24 h, temsirolimus was added for 3 d and then washed out. Controls included cells exposed to the diluents for docetaxel and/or temsirolimus, with similar washes. After treatment, fresh medium was added and cells were harvested on day 7. Cells were counted and serial dilutions were plated to determine cell survival in a colony-forming assay.

Human breast cancer MDA-468 cells were treated with either 5-FU or temsirolimus, or both in combination (or their diluents), using the same schedule as for PC-3 and LnCaP cells.

Cell cycle analysis. Cell cycle analysis was done by flow cytometry. Cells that were exposed to various doses of temsirolimus for 3 d were harvested after exposure, and cells treated with concurrent or sequential scheduling, were harvested on day 7 and fixed in 80% ethanol on ice. All cell samples were kept in a -20°C freezer until flow cytometry was done. Once cells were taken out of the freezer, 1 mL of cold PBS was added and samples were centrifuged. After washing with cold PBS twice, the cells were then treated with 0.5 mL propidium iodide/RNase Staining Buffer (BD Biosciences Pharmingen) for at least 6 h. Samples were analyzed for cell cycle distribution on a FACScan (Becton Dickinson) using the Cell Quest software.

Concurrent or sequential treatment of xenografts. To generate xenografts, PC-3 cells (2×10^6) and LnCaP cells (4×10^6 in 0.1 mL Matrigel) were injected s.c. into both flanks of male nude mice, and MDA-468 cells (2×10^6) were injected similarly in female nude mice. All animals

Fig. 1. Sequence of chemotherapy and temsirolimus (CCI-779) used (*A*) *in vitro* and (*B*) in nude mice bearing xenografts. The sequence was repeated weekly for 3 wk for treatment of xenografts.
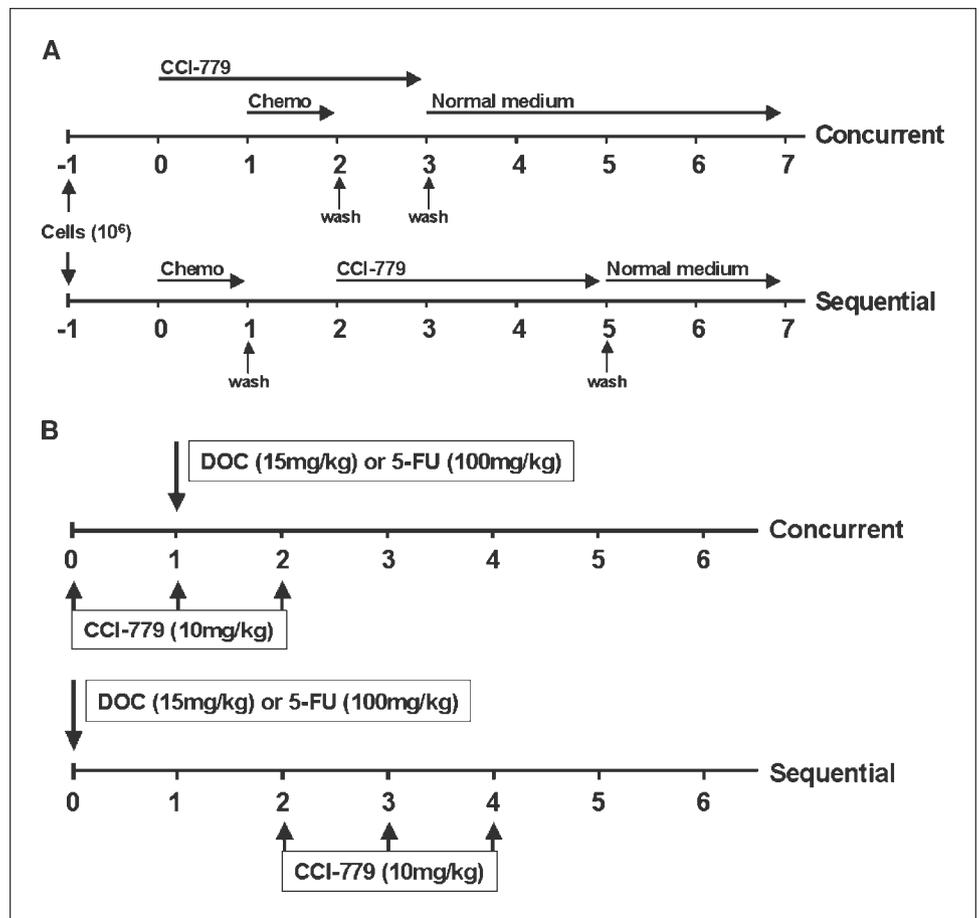
were tagged and sorted randomly into groups (as described below), and each group had at least 10 tumors. Once tumors reached a size of ~50 mm³, treatments were initiated as follows; the first day of treatment was called day 0. When treatments were completed, the animals were regrouped to avoid observer bias. Tumor volumes were plotted as a function of time following initiation of treatment. All experiments were repeated.

Different groups of mice received the following treatments (Fig. 1B): (*a*) control: vehicle solution 0.1 mL i.p.; (*b*) temsirolimus alone: 10 mg/kg i.p. 3 consecutive d per week for 3 wk on days 0 to 2, 7 to 9, and 14 to 16; (*c*) docetaxel or 5-FU alone: docetaxel 15 mg/kg or 5-FU 100 mg/kg i.p. once weekly for three doses on days 0, 7, and 14; (*d*) docetaxel or 5-FU plus temsirolimus (concurrent): docetaxel or 5-FU on days 1, 8, and 15, with temsirolimus on days 0 to 2, 7 to 9, and 14 to 16; (5) docetaxel or 5-FU followed by temsirolimus (sequential): docetaxel or 5-FU on days 0, 7, and 14, and temsirolimus on days 2 to 4, 9 to 11, and 16 to 18.

Results

Effects of temsirolimus and chemotherapy **in vitro.** The growth of PC-3, LnCaP, and MDA-468 cells was inhibited by temsirolimus in a dose-dependent manner (Fig. 2A). PC-3 and LnCaP cells were slightly more sensitive to temsirolimus than MDA-468 cells, especially at the lowest concentration of 0.1 μmol/L. There was no significant difference at higher concentrations. Close to maximum suppression of growth for all cell lines was obtained following exposure to 0.5 μmol/L temsirolimus.

Using a colony-forming assay, the mean surviving fractions (±SEM) of PC-3 cells after 24-h treatment with 5 or 10 nmol/L docetaxel were found to be 38 ± 9% and 21 ± 8%, respectively. LnCaP cells were more responsive to 24-h treatment with the same doses of docetaxel, with surviving fractions of 6 ± 5% and 0.2 ± 0.1%, respectively. The surviving fractions of MDA-468 cells after 24-h treatment with 10 or 50 μmol/L 5-FU were 65 ± 4% and 15 ± 4%, respectively.

Cell cycle analysis showed that for all three cell lines the percentage of G_1 phase cells increased after a 3-d treatment with temsirolimus (0.5 μmol/L), whereas the percentage of S phase cells decreased (Fig. 2B-D). The percentage of G_2-M phase cells did not change significantly. There was no significant difference in the cell cycle distribution of PC-3 or LnCaP cells on day 7 following temsirolimus alone or either concurrent or sequential scheduling with docetaxel and temsirolimus, or for 5-FU and temsirolimus with MDA-468 cells ($P > 0.05$ in each group; Fig. 3A-C).

The numbers of reproductively viable PC-3 and LnCaP cells on day 7 after various treatments, as determined by a colony-forming assay, are shown in Fig. 4A and B, respectively; there was no significant difference between the sequential or concurrent scheduling groups, or between combined treatment and docetaxel alone (Fig. 4A and B).

There was no significant difference in the number of colony-forming MDA-468 cells treated with 0.5 μmol/L temsirolimus and 10 μmol/L 5-FU concurrently or sequentially, and no effect to reduce the number below controls (Fig. 4C).
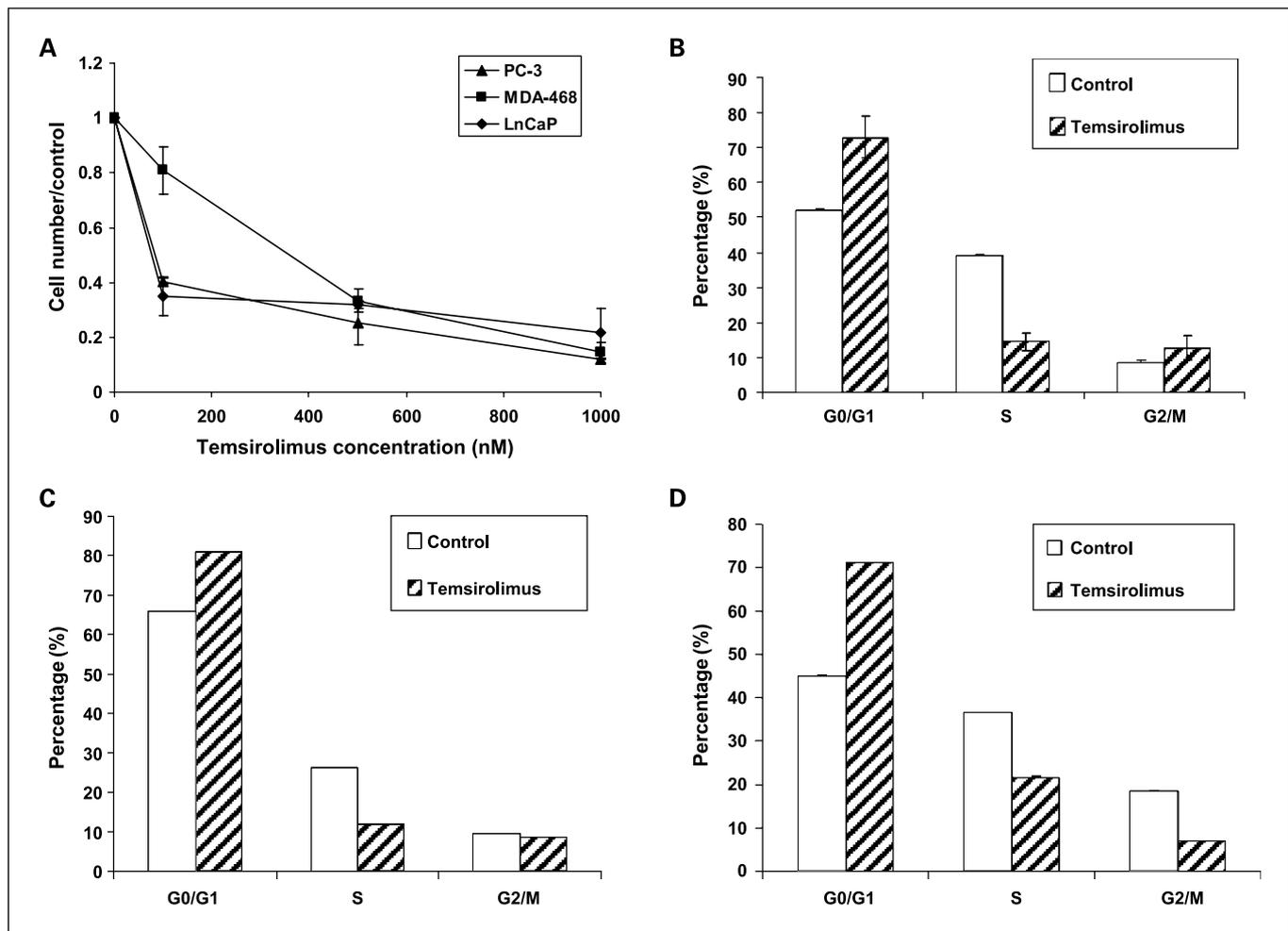
Fig. 2. Effect of 3 d of treatment with temsirolimus on (*A*) number of PC-3 (▲), LnCaP (♦), and MDA-468 (■) cells (compared with control cultures treated with diluent). Cell cycle analysis of (*B*) PC-3, (*C*) LnCaP, and (*D*) MDA-468 cells after 3-d exposure to 0.5 μmol/L temsirolimus. *Columns*, mean of three independent experiments; *bars*, SEM.

Effects of chemotherapy and temsirolimus on xenografts. Growth inhibition of prostate cancer PC-3 xenografts following various treatments is shown in Fig. 5A. There was no observable difference in mean tumor size between concurrent and sequential treatment until day 28, but concurrent scheduling was more effective in delaying subsequent regrowth of the xenografts. Mean tumor volume at day 52 was 167 ± 32 mm^3 and 461 ± 114 mm^3 for concurrent and sequential treatment, respectively ($P = 0.038$). Both combined treatment groups showed significantly greater tumor growth delay than treatment with temsirolimus alone, docetaxel alone, and especially when compared with controls.

LnCaP xenografts were difficult to grow in nude mice; and once established, the tumors grew slowly. There was no effect of temsirolimus treatment alone to influence the growth of LnCaP xenografts, whereas docetaxel treatment had small effects to inhibit tumor growth. Concurrent but not sequential administration of docetaxel and temsirolimus had greater effects to cause tumor shrinkage and inhibit tumor regrowth than docetaxel alone (data not shown).

MDA-468 tumors grew quite slowly in nude mice, and there were relatively small effects of 5-FU alone, temsirolimus alone, or combined treatment to increase growth delay (Fig. 5B). There

was no significant difference between the effects of concurrent and sequential treatment. No significant differences in body weight were observed between groups, suggesting limited toxicity with combined treatment.

Discussion

Our previous study showed that the PTEN-negative human prostate cancer PC-3 cell line is more sensitive to treatment with temsirolimus than the DU145 prostate cancer cell line with wild-type PTEN (14). Therefore, the PC-3 tumor line was used in the present study to compare concurrent and sequential treatments with temsirolimus and docetaxel (the preferred drug for treatment of human prostate cancer; refs. 21, 22) in cells and tumor xenografts, and we also tested the PTEN-mutant human prostate cancer cell line, LnCaP (23). The human breast cancer MDA-468 cell line was selected for this study because it is reported to be PTEN negative and sensitive to temsirolimus, with an IC$_{50}$ in the nanomolar range, due to overexpression of S6K1 and expression of phosphorylated Akt/phosphoinositide 3-kinase/Akt (8, 24). In preliminary experiments we confirmed that the MDA-468 cell line was more sensitive to temsirolimus than other breast cancer cell lines, such as MDA-231, MDA-435,

and MCF-7. However, temsirolimus was more effective against human prostate cancer PC-3 cells and xenografts than human breast cancer MDA-468 cells and xenografts when used alone or in combination with chemotherapy. Temsirolimus also led to greater growth delay of PC-3 xenografts than docetaxel alone; this effect is greater than that expected from the *in vitro* sensitivity, and differs from the general experience in treatment of human prostate cancer, where chemotherapy has shown greater activity than molecular-targeted agents. In contrast, for LnCaP cells, temsirolimus had similar inhibitory effects to those observed with PC-3 cells in culture, but no significant effect on LnCaP xenografts.
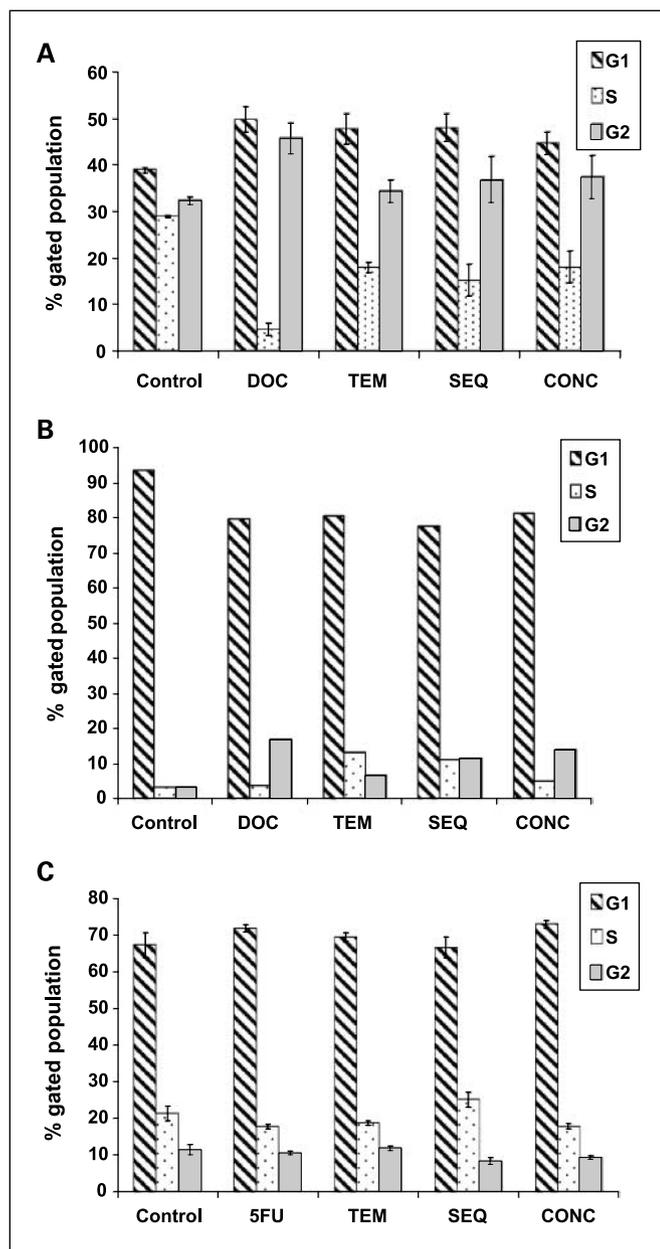

Fig. 3. Cell cycle analysis of (*A*) PC-3, (*B*) LnCaP, and (*C*) MDA-468 cells on day 7 after sequential or concurrent treatment with 10 nmol/L docetaxel (*DOC*) or 10 μmol/L 5-FU and 0.5 μmol/L temsirolimus (*TEM*). *Columns*, means of three independent experiments; *bars*, SEM.
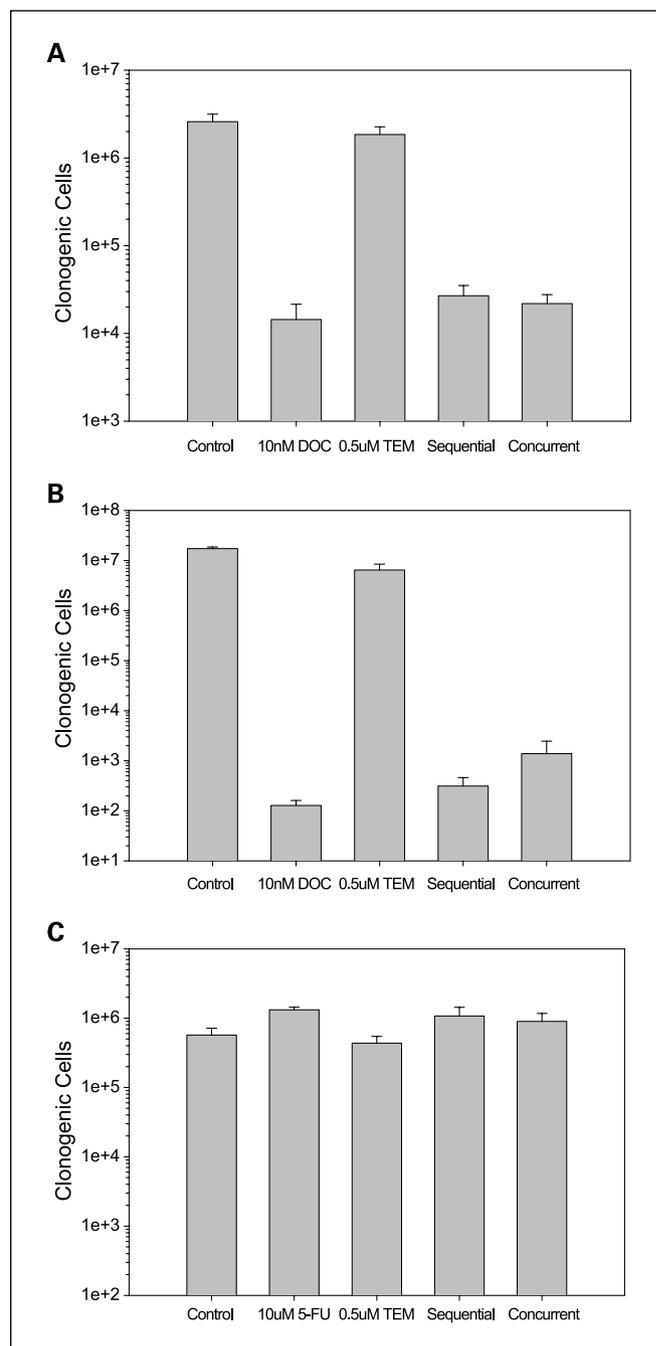

Fig. 4. Effects of concurrent and sequential scheduling of chemotherapy and temsirolimus (see Fig. 1A for schedules) on number of colony-forming cells present at day 7 in cultures of (*A*) PC-3, (*B*) LnCaP, and (*C*) MDA-468 cells. *Columns*, mean of four independent experiments; *bars*, SEM.

Concurrent administration of temsirolimus and docetaxel led to better outcome than sequential scheduling for PC-3 tumors and for LnCaP tumors, but there was no significant difference between the two treatment schedules for the MDA-468 tumors. We hypothesized that temsirolimus administered sequentially between docetaxel treatments would be more effective at inhibiting repopulation compared with concurrent treatment, mainly due to effects of the inhibitor on cell cycle. Temsirolimus puts cells out of cycle (G_1 growth arrest); therefore, concomitant
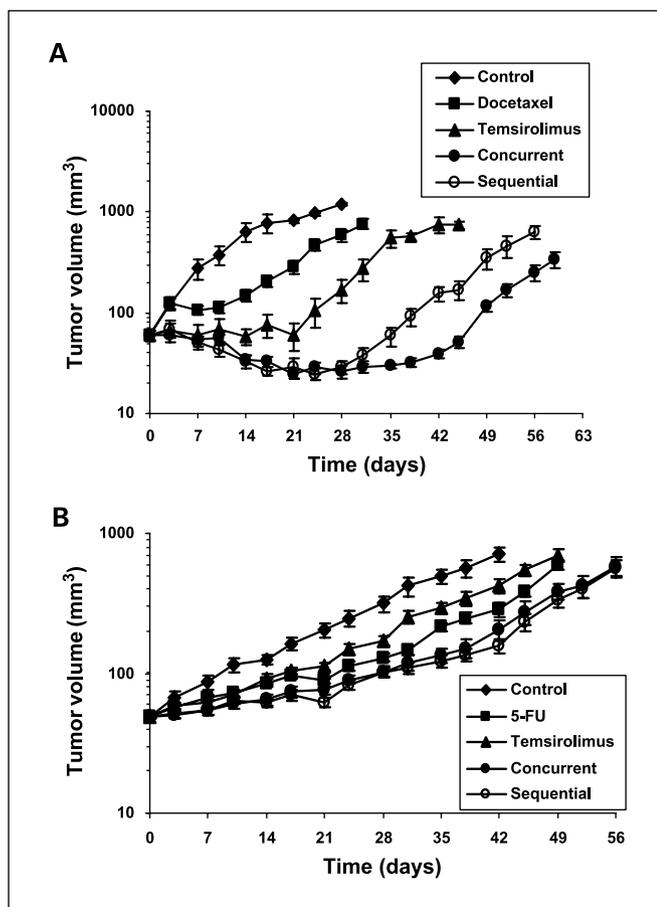
Fig. 5. Effects of concurrent and sequential schedules of chemotherapy and temsirolimus on growth of (*A*) PC-3 xenografts (chemotherapy = docetaxel) and (*B*) MDA-468 xenografts (chemotherapy = 5-FU). Both plots represent one experiment, with repeat experiments showing similar trends; error *bars*, SEM.

administration of temsirolimus with docetaxel may decrease the efficacy of cycle-active chemotherapy. However, our *in vitro* results showed no difference in cell cycle distribution between sequential and concurrent combined treatment on day 7, before the subsequent course of chemotherapy in PC-3 cells and LnCaP cells (Fig. 3). The distribution of the S-phase population in the sequential treatment group is similar to that observed on day 7 following temsirolimus treatment alone, suggesting that the effects of temsirolimus might have been dominant at this time with the combined treatment (Fig. 3). Docetaxel treatment led to a lower S-phase population and a slight increase in the G_1 and G_2-M populations compared with both combined treatment groups (Fig. 3). Growth arrest at G_1 and G_2-M has been previously observed following taxane treatment (ref. 25; and data from our laboratory). This result suggests that docetaxel treatment continued to have effects on cell cycle at day 7 following treatment; however, this did not translate into a difference in survival as indicated by similar clonogenic cell numbers in the docetaxel alone and combined treatment groups (Fig. 4A-B). In addition, a higher sub-G_0/G_1 population, which has been shown to represent apoptotic cells, was observed following docetaxel alone compared with sequential or concurrent combined treatment (data not shown), which might also account for the differences in cell cycle distribution. It is not clear why gefitinib might

have changed the effects on cell cycle of docetaxel in the combined treatment groups.

Despite similar cell cycle effects between concurrent and sequential treatments in our *in vitro* experiments, *in vivo* growth delay studies showed better delay of tumor regrowth with concurrent temsirolimus and docetaxel treatment compared with sequential treatment in the prostate xenografts. Preclinical studies and clinical trials that have compared sequential versus concurrent administration of chemotherapy with tamoxifen or inhibitors of the epidermal growth factor receptor have favored sequential treatment (16–20). We had hypothesized that sequential scheduling might be superior because of inhibition of repopulation by temsirolimus between courses of chemotherapy. However, it is possible that the main effects of combined treatment on tumor repopulation were not due to cell cycle factors, but rather effects of treatment on the tumor microenvironment.

Studies have suggested that both docetaxel and temsirolimus have antiangiogenic properties (26, 27). Sweeney and colleagues (26) showed that docetaxel inhibited endothelial cell growth and capillary formation *in vitro* in a dose-dependent manner. Studies in our laboratory have shown antiangiogenic effects of paclitaxel (a related taxane) *in vivo*,[1] and ongoing studies in our laboratory are evaluating the effects of docetaxel on tumor vasculature in human prostate xenografts. Temsirolimus may inhibit tumor growth through antiangiogenic mechanisms associated with the targeting of the mTOR/hypoxia-inducible factor-1α/vascular endothelial growth factor signaling pathway, as indicated by decreased levels of hypoxia-inducible factor-1α, vascular endothelial growth factor expression, and microvessel density (27). A decrease in tumor vasculature would likely lead to less repopulation within tumors and increased cell death.

As temsirolimus was the dominant treatment in our studies with PC-3 xenografts, cells may have remained out of cycle when the second and third docetaxel doses were given in the sequential schedule. Our *in vivo* results suggest that three daily doses of temsirolimus given thrice at weekly intervals are able to completely abrogate growth of PC-3 xenografts for 21 days, whereas effects against single cells in culture seem to be more transient. The effects of temsirolimus and docetaxel to delay tumor growth seem to be at least additive, especially for the concurrent schedule; therefore, for treatment of PC-3 xenografts, the dominant effect of temsirolimus may have been to up-regulate mechanisms leading to cell death (28).

There was a slight delay in the regrowth of MDA-468 breast cancer xenografts following combined treatment compared with treatment with either agent alone (Fig. 5B); however, the effects were not as prominent as those observed with PC-3 xenografts (Fig. 5A). The minimal effect of temsirolimus alone on MDA-468 cells and xenografts might be attributed to changes in signaling events following mTOR inhibition: a study by Sun et al. (29) showed that treatment with rapamycin (an inhibitor of mTOR) in various cancer cell lines led to a decrease in the amount of phosphorylated p70S6K (a downstream marker of mTOR activity), which is indicative of mTOR inhibition; however, rapamycin also induced a subsequent increase in p-Akt and p-eIF4E levels following treatment. These results were attributed to a feedback mechanism associated with phosphoinositide 3-kinase–dependent activation of the Akt and eIF4E proteins

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[1] Unpublished data.

(29), which would likely result in downstream signaling events leading to cell survival and proliferation.

Temsirolimus has been studied extensively in clinical trials as a single agent (30); however, there are limited studies of the combination of temsirolimus and chemotherapy. A study of temsirolimus administered concomitantly with gemcitabine for the treatment of pancreatic cancer showed that combination therapy was more effective at inhibiting tumor growth than either agent alone (31). A phase I trial testing the effect of temsirolimus and 5-FU and leucovorin in patients with advanced solid tumors showed partial tumor responses in 3 of 26 patients; however, the study was stopped due to high toxicity observed in patients in the combined treatment arm (32). In that study, treatment with temsirolimus was initiated on day 8, before leucovorin/5-FU (32).

Our study shows that combined docetaxel and temsirolimus treatment is more effective at delaying tumor regrowth than either agent alone in human prostate tumors. In addition, combined treatment administered concurrently was more efficacious than sequential administration of these agents for both PC-3 and LnCaP xenografts. Clinical trials should evaluate optimal dosing and scheduling of combined chemotherapy and growth inhibitor treatment. Our study suggests that combined docetaxel and temsirolimus treatment might be beneficial in the treatment of men with prostate cancer.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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