Bevacizumab plus 5-fluorouracil induce growth suppression in the CWR-22 and CWR-22R prostate cancer xenografts

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Abstract
Prostate cancer is the most common malignancy in men. Although patients with metastatic prostate cancer can benefit from androgen ablation, most of them will die of prostate cancer progression to an androgen-refractory state. In the present study, the effects of docetaxel, bevacizumab, 5-fluorouracil (5-FU), bevacizumab plus docetaxel, and bevacizumab plus 5-FU on the growth of human CWR-22 (androgen-dependent) and CWR-22R (androgen-independent) prostate carcinoma xenografts were investigated. We report that i.p. administration of 10 mg/kg docetaxel at 1-week interval, 5 mg/kg bevacizumab once every 2 weeks, or 12.5 mg/kg 5-FU, bevacizumab/docetaxel, or bevacizumab/5-FU weekly to severe combined immunodeficient mice bearing prostate cancer xenografts (12 mice per treatment group) for 21 days resulted in 22.5 ± 8%, 23 ± 7%, 31 ± 8%, 22 ± 6%, and 81 ± 5% growth inhibition, respectively. Greatest growth suppression was observed in bevacizumab/5-FU treatment. Bevacizumab/5-FU–induced growth suppression was associated with reduction in microvessel density, inhibition of cell proliferation; up-regulation of phosphatase and tensin homologue, p21 Cip1/Waf1, p16 INK4a, and p27 Kip1; hypophosphorylation of retinoblastoma protein; and inhibition of Akt/mammalian target of rapamycin pathway. Our data indicate that bevacizumab/5-FU effectively inhibits angiogenesis and cell cycle progression and suggest that bevacizumab/5-FU may represent an alternative treatment for patients with prostate cancer. [Mol Cancer Ther 2007;6(8):2149–57]

Introduction
Prostate cancer represents the most common disease in men worldwide and is the second cause of death from malignant cancers (1, 2). The clinical outcome of prostate cancer is strongly correlated to its differentiation and tumor grade (3). Whereas patients with localized disease may be cured with surgery or radiation, most patients respond initially to ablation of gonadal androgen production through either orchiectomy or luteinizing hormone–releasing hormone agonists (4). Eventually, most patients will develop progressive disease despite continued androgen suppression. The main options currently available for hormone-refractory prostate cancer are second-line hormonal manipulations, radiation therapy, traditional cytotoxic chemotherapy, and investigational therapy with novel and targeted agents (5). Currently, docetaxel plus prednisone is a standard second-line therapy for hormone-refractory prostate cancer patients (reviewed in ref. 6).

Prostate cancer arises from multiple genetic and epigenetic aberrations (7). Common aberrations in human prostate cancer are reduced expression of pRb (8) and frequent inactivation of phosphatase and tensin homologue (PTEN; ref. 9). The degree of PTEN deficiency is closely correlated with activation of the oncogenic kinase Akt (10). Akt activation affects cell cycle progression through control of phosphorylation of eukaryotic translation initiation factor 4E and mRNA translation via control of phosphorylation of eukaryotic translation initiation factor 4E–binding protein 1 (4E-BP1) and its dissociation from the mRNA cap binding protein eukaryotic translation initiation factor 4E (11). Targeting of activated Akt to mouse prostate induces prostate intraepithelial neoplasia, which is reversed following administration of the mammalian target of rapamycin (mTOR) inhibitor RAD001 (12). It has been reported that the growth and proliferation of tumors expressing constitutive activation of phosphatidylinositol 3-kinase and Akt or inactivation of PTEN displayed enhanced sensitivity to mTOR inhibitors (reviewed in ref. 13). Recent studies have found that mTOR activation is also important for the secretion of vascular endothelial growth factor (VEGF; ref. 14).

Prostate cancer specimens show increased VEGF expression and vascularity when compared with benign prostatic hyperplasia and normal prostate tissue (15). An association does exist between microvessel density in tumor, Gleason score, metastases, tumor aggressiveness, and progression (16–18). VEGF levels correlate with disease stage and perhaps survival in the metastatic setting and hormone-refractory prostate cancer (19). In the CWR-22 prostate cancer xenograft model, the addition of paclitaxel enhances the antitumor activity of monoclonal VEGF antibody (20). The safety and efficacy of bevacizumab/docetaxel/
estramustine combination for hormone-refractory prostate cancer (reviewed in ref. 21) and of bevacizumab/irinotecan/5-fluorouracil (5-FU)/leucovorin combination for metastatic colorectal cancer (22) have been reported. Several antiangiogenic agents are under investigation in clinical trials for prostate cancer, including thalidomide (23), thalidomide analogues (24, 25), and VEGF receptor inhibitors such as AZD6474, KRN633, and CEP-7055 (reviewed in ref. 26).

In the present study, we show that addition of bevacizumab to 5-FU–based chemotherapy potently inhibits tumor growth, angiogenesis, cell cycle progression, and the Akt/mTOR pathway. This combination holds a promise as an adjuvant to conventional therapies.

Materials and Methods

Docetaxel (Taxotere) was obtained from Aventis Laboratories. Fluorouracil Injection B.P. was from Mayne Pharma Plc. Bevacizumab was from Genentech, Inc. Antibodies against pRB, α-tubulin, cyclin A, cyclin B1, cyclin D1, Cdk-4, Cdk-2, p21Cip1/Waf1, p16INK4a, and p27Kip1 were obtained from Santa Cruz Biotechnology, Inc. Phosphorylation-specific antibodies against mTOR (Ser2448), p70S6 kinase (Thr422/Ser424), p70S6 kinase (Thr389), 4E-BP1 (Ser37/40), 4E-BP1 (Thr70), Akt (Ser308), Akt (Thr421/Ser424), p70S6 kinase (Thr389), S6R (Ser235/236), S6R (Thr421/Ser422), 4E-BP1 (Ser24/46), 4E-BP1 (Thr37), Akt (Ser208), Akt (Ser473), pRB (Ser807/S811), and pRB (Ser856) and antibodies against 4E-BP1, p70S6 kinase, and mTOR were obtained from Cell Signaling Technology. Antibodies against PTEN, CD31/platelet endothelial cell adhesion molecule-1, and Ki-67 were obtained from NeoMarkers. Antibodies against 4E-BP1, p70S6 kinase, and mTOR were obtained from Santa Cruz Biotechnology, Inc. Phosphorylation-specific antibodies against mTOR (Ser2448), p70S6 kinase (Thr422/Ser424), p70S6 kinase (Thr389), 4E-BP1 (Ser37/40), 4E-BP1 (Thr70), Akt (Ser208), Akt (Ser473), pRB (Ser807/S811), and pRB (Ser856) and antibodies against 4E-BP1, p70S6 kinase, and mTOR were obtained from Cell Signaling Technology. Conjugated secondary antibodies were supplied by Pierce. The chemiluminescent detection system was supplied by Amersham Pharmacia Biotech.

Tumorigenicity in Severe Combined Immunodeficient Mice

The study received ethics board approval from the National Cancer Centre of Singapore and Singapore General Hospital. All mice were maintained according to the “Guide for the Care and Use of Laboratory Animals” published by NIH. They were provided with sterilized food and water ad libitum and housed in negative pressure isolators with 12-h light/dark cycles.

Prostate cancer xenografts were carried out with male severe combined immunodeficient (SCID) mice of 9 to 10 weeks of age (Animal Resources Centre, Canning Vale, West Australia). Androgen-dependent human CWR-22 (27) and androgen-independent CWR-22R (28) prostate cancer xenografts were miniced under sterile conditions. Approximately 1 × 10^7 cells were s.c. implanted in both flanks of male SCID.

To investigate the effects of bevacizumab, docetaxel, and 5-FU on the growth of prostate cancer xenografts, these drugs were diluted in saline solution at an appropriate concentration. Mice bearing tumor xenografts were divided into five groups and each consisted of 12 mice. They were i.p. injected with 100 μL of saline, 5 mg/kg bevacizumab every 2 weeks (29), or weekly with 12.5 mg/kg 5-FU (30), 10 mg/kg docetaxel (two doses in total at one-week interval as described; ref. 31), bevacizumab plus 5-FU, or bevacizumab plus docetaxel for 21 days. Treatments started on day 7 after tumor implantation. By this time, the tumors had reached the size of ~100 mm^3. Tumor growth was monitored every 2 days by vernier caliper measurement of the length (a) and width (b) of the tumor. Tumor volume was calculated as (a × b^2)/2. The animals were sacrificed on day 21 during the treatment because the tumor size in the controls exceeded 1,500 mm^3 in accordance with the animal care protocol. Body weight and tumor weight were recorded, and tumors harvested for later analysis.

Efficacy of antitumor agents was determined by %T/C, where T and C are the median tumor weight (in milligrams) of drug-treated and vehicle-treated mice at day 21 during the treatment, respectively. Ratios of ≤42% are considered an active response (Drug Evaluation Branch of the Division of Cancer Treatment, National Cancer Institute criteria).

Immunohistochemistry

Five-micrometer sections were dewaxed, rehydrated, and subjected to antigen retrieval. After blocking endogenous peroxidase activity and nonspecific staining, the sections were incubated with antibodies against Ki-67, CD31, and cleaved caspase-3 (overnight at 4°C). Immunohistochemistry was done using the streptavidin-biotin peroxidase complex method according to the manufacturer’s instructions (Lab Vision) using 3,3′-diaminobenzidine as the chromogen. Sections known to stain positively were incubated in each batch and negative controls were also prepared by replacing the primary antibody with preimmune sera. For Ki-67, only nuclear immunoreactivity was considered positive. The number of labeled cells among at least 500 cells per region was counted and then expressed as percentage values. For the quantification of mean vessel density in sections stained for CD31, 10 random 0.159-mm^2 fields at ×100 magnification were captured for each tumor and microvessels were quantified. The data were expressed as mean ± SE.

Western Blot Analysis

To determine changes in indicated proteins, independent tumors from vehicle-treated (n = 3), bevacizumab-treated (n = 3–4), 5-FU–treated (n = 2–3), and bevacizumab/5-FU–treated (n = 4) mice were homogenized in buffer containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L l-glycerolphosphate, 2 mmol/L Na3VO4, 1 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride. One hundred micrograms of tissue lysate were subjected to Western blot analysis as previously described (30). Blots were incubated with indicated primary antibodies and 1:7,500 dilution of secondary antibodies. All primary antibodies were used at a final concentration of 1 μg/mL. The blots were then visualized with a chemiluminescent detection system (Amersham) as described by the manufacturer.
Figure 1. Effects of docetaxel, bevacizumab, 5-FU, bevacizumab plus docetaxel, and bevacizumab plus 5-FU treatments on body weight at sacrifice, growth rate, and tumor weight of CWR-22 and CWR-22R xenografts. The indicated xenografts were s.c. implanted on the right side of male SCID mice as described in Materials and Methods. Mice bearing hepatocellular carcinoma xenografts were randomized to one of the four treatment groups (12 mice per group) and treated with either vehicle, 10 mg/kg docetaxel, or 5 mg/kg bevacizumab (BEV), 12.5 mg/kg 5-FU, bevacizumab plus docetaxel, or bevacizumab plus 5-FU for 21 d starting from day 7 after tumor implantation as described in Materials and Methods. A, body weight at sacrifice (day 21 during treatment). B, tumor volume at a given time for CWR-22R xenografts treated with various treatments. C, efficacy (% T/C) of tested drugs. Ratios of ≤42% are considered an active response (Drug Evaluation Branch of the Division of Cancer Treatment, National Cancer Institute criteria). Experiments were repeated at least thrice with similar results. Columns with different letter indicate significant differences at $P < 0.01$. 

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Statistical Analysis  
To obtain the $P$ values, the experiments were repeated thrice. Differences in tumor growth and expression of indicated proteins, tumor weight, Ki-67 index, mean vessel density, and cleaved caspase-3–positive cells were analyzed by ANOVA.

Results  
To study the effects of docetaxel, bevacizumab, 5-FU, and their combinations on prostate cancer growth, mice bearing CWR-22 and CWR-22R xenografts were treated with docetaxel, bevacizumab, 5-FU, bevacizumab/docetaxel, and bevacizumab/5-FU. Both animal toxicity and the ability of these treatments to inhibit tumor formation and progression were determined. In preliminary studies, we found that treatment with a nonspecific antibody of the same immunoglobulin G isotype had no effect on tumor growth and was essentially equivalent to vehicle alone (data not shown). No overt toxicity of docetaxel, bevacizumab, 5-FU, and the combined therapies was observed during the course of treatment as defined by weight loss, unkempt appearance or mortality, and behavior (Fig. 1A). The efficacy of docetaxel, bevacizumab, and 5-FU as determined by $\%T/C$ is shown in Fig. 1C. All the treatments significantly inhibited tumor growth ($P < 0.01$). Figure 1C shows that $T/C$ ratio in bevacizumab/5-FU was $<0.42$ (Drug Evaluation Branch of the Division of Cancer Treatment, National Cancer Institute criteria), suggesting that this combination was very active. The androgen-dependent human CWR-22 was more sensitive than androgen-independent CWR-22R prostate cancer (Fig. 1C). Because bevacizumab/5-FU combination exhibits higher antitumor activity than bevacizumab/docetaxel, this combination was selected for further studies.

We next examined the association between the antitumor activity of bevacizumab, 5-FU, and bevacizumab/5-FU treatments and their ability to inhibit blood vessel formation in prostate cancer xenografts. Bevacizumab (Fig. 2C) and bevacizumab/5-FU (Fig. 2D) decreased blood vessels supplied to the tumors. Mild suppression of 5-FU on neovascularization was also noticed (Fig. 2B). Table 1 shows the median number of CD31-positive tumor cells from vehicle-, bevacizumab-, 5-FU–, and bevacizumab/5-FU–treated tumors on day 21 during the treatment. The number of microvessels in bevacizumab-treated tumors was approximately 35% and 17% of that seen in vehicle-treated tumors (Table 1). Although bevacizumab/5-FU potently inhibited angiogenesis, we observed very little necrosis in bevacizumab/5-FU–treated tumors (data not shown).

Figure 2. Effects of bevacizumab, 5-FU, and bevacizumab plus 5-FU therapies on neovascularization of CWR-22R prostate cancer xenografts. CWR-22R cells were s.c. implanted on both flanks of male SCID mice as described in Materials and Methods. Mice bearing hepatocellular carcinoma xenografts were randomized to one of the four treatment groups (12 mice per group) and treated with either vehicle (A), 5-FU (B), bevacizumab (C), or bevacizumab/5-FU (D) for 21 d starting from day 7 after tumor implantation as described in Materials and Methods. Representative pictures of blood vessel supply to the tumors at sacrifice. Experiments were repeated thrice with similar results. Note that bevacizumab/5-FU inhibited the formation of blood vessels thereby cutting blood supply to the tumor cells.
To examine the antiproliferative and apoptotic effects of bevacizumab, 5-FU, and combined bevacizumab/5-FU treatments in vivo, sections were stained with Ki-67 and cleaved caspase-3 antibodies. Table 1 shows that the Ki-67 labeling index in bevacizumab-, 5-FU-, and bevacizumab/5-FU–treated tumors was significantly decreased compared with vehicle-treated tumors (P < 0.01). Further decrease in number of cells stained with Ki-67 antibody was observed in the combined therapy. The percentage of cells stained for cleaved caspase-3 was significantly increased in 5-FU–treated (P < 0.01) but not in bevacizumab– or bevacizumab/5-FU–treated tumors, suggesting that bevacizumab and bevacizumab/5-FU did not cause any significant apoptosis (Table 1).

Because PTEN deficiency leads to Akt activation and triggers initiation of prostate cancer (9), we determined whether bevacizumab/5-FU–inhibited prostate cancer xenograft growth was associated with changes in these proteins. Figure 3 shows that whereas 5-FU had no effect on PTEN expression and Akt phosphorylation, bevacizumab slightly increased phosphorylation of Akt at Ser473. On the other hand, PTEN expression in bevacizumab/5-FU–treated tumors was increased by 3.6-fold (P < 0.01). Total pRb was not significantly decreased in the past decades because prostate cancer cells are barely responsive to chemotherapeutic agents or radiotherapy (reviewed in ref. 6). Although patients with metastatic prostate cancer can benefit from androgen ablation, most of them will die of prostate cancer progression to an androgen-refractory state. Therefore, an effective treatment strategy against prostate cancer is needed to spare the burden of the patients. In the present study, we show that bevacizumab, docetaxel, and 5-FU, when given as single agents, inhibit the growth of both androgen-dependent and androgen-independent prostate cancer xenografts. The growth of prostate cancer xenografts is further suppressed when bevacizumab is given with 5-FU but not docetaxel. Bevacizumab/5-FU up-regulates 4E-BP1, mTOR, and p70S6 kinase was not altered by any treatments, suggesting that the mTOR pathway is inactivated. Figure 4 shows that a slight increase in cyclin D1 was seen in bevacizumab-treated samples. Cyclin B1, cyclin A, Cdk-2, and Cdk-4 expression was not affected by 5-FU or bevacizumab treatment. The levels of positive regulators Cdk-2 and Cdk-4 in bevacizumab/5-FU–treated tumors were significantly decreased (P < 0.01). Up-regulation of p21Waf/p16ink4a, and p27kip1 and inhibition of pRb phosphorylation at Ser807/811 were seen only in bevacizumab–5-FU–treated tumors. Whereas total pRb was not significantly altered, hypophosphorylation of pRb at Ser780 was significantly reduced across the treatments. Similar results were obtained when bevacizumab/5-FU–treated CWR-22 tumors were analyzed (Supplementary data).1

### Discussion

Despite tremendous efforts and resources devoted to treatment, the incidence and mortality of prostate cancer have not decreased in the past decades because prostate cancer cells are barely responsive to chemotherapeutic agents or radiotherapy (reviewed in ref. 6). Although patients with metastatic prostate cancer can benefit from androgen ablation, most of them will die of prostate cancer progression to an androgen-refractory state. Therefore, an effective treatment strategy against prostate cancer is needed to spare the burden of the patients. In the present study, we show that bevacizumab, docetaxel, and 5-FU, when given as single agents, inhibit the growth of both androgen-dependent and androgen-independent prostate cancer xenografts. The growth of prostate cancer xenografts is further suppressed when bevacizumab is given with 5-FU but not docetaxel. Bevacizumab/5-FU up-regulates

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1 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
PTEN, an upstream down-regulator of phosphatidylinositol 3-kinase/Akt, and inhibits the phosphorylation of Akt, an upstream positive modulator of mTOR, leading to inactivation of p70S6 kinase and 4E-BP1. In addition, bevacizumab/5-FU also enhances the expression of p21\(^{G_{1}p1}\)/P27\(^{kip1}\), p16\(^{INK4a}\), and p27\(^{Kip1}\), resulting in hypophosphorylation of retinoblastoma. These events may contribute to its potent growth inhibition. By inhibiting protein synthesis, cell cycle progression, and angiogenesis, bevacizumab/5-FU therapy may prove to be useful in maintaining dormancy of micrometastasis and preventing the development of overt recurrence or metastasis after surgical resection of a primary tumor.

Both 5-FU and bevacizumab, when administered as single agents, reduced but did not fully suppress the growth of CWR-22R and CWR-22 prostate cancer xenografts. However, bevacizumab/5-FU significantly suppressed prostate cancer growth to a greater degree than single-agent therapy. The mechanism of action of bevacizumab/5-FU is likely to be multifactorial and should be further investigated using different approaches. Recently, studies have discovered that the mTOR pathway regulates tumor angiogenesis as it stimulates VEGF production by tumor cells (35). It is possible that combined suppression of both VEGF protein (by bevacizumab) and VEGF expression (by targeting mTOR via bevacizumab/5-FU) might induce a synergistic inhibition of prostate tumor angiogenesis. Thus, one function of bevacizumab/5-FU is to prevent the tumor mass from expanding by preventing further development of the tumor neovascular network.

![Figure 3. Effects of bevacizumab, 5-FU, and bevacizumab plus 5-FU therapies on the expression of proteins involved in the PTEN/Akt/mTOR pathway in CWR-22R xenografts. CWR-22R cells were implanted and treated as described in Fig. 2. Lysates from vehicle- and drug-treated tumors were subjected to Western blot analysis as described in Materials and Methods. Blots were incubated with the indicated antibodies. Each lane represents the tumor from one individual mouse from the same experiment. Changes in the expression of the indicated proteins are shown. Experiments were repeated at least thrice with similar results.](image-url)
reported that tumor-associated endothelial cells are targets of bevacizumab in vivo. These cells express VEGF receptor and require VEGF for proliferation and survival (36). With the inhibition of activity by bevacizumab and VEGF production by bevacizumab/5-FU through inhibition of mTOR, tumor-associated endothelial cells, whose proliferating frequency is 20 to 2,000 times higher than that of endothelial cells in normal organs (37, 38), would be more sensitive to the combined bevacizumab/5-FU treatment. Supporting this hypothesis, we observe that bevacizumab/5-FU–treated tumors have a gross reduction in blood supply (Fig. 2D) and fewer visible blood vessels compared with bevacizumab and 5-FU.

We have shown that docetaxel, when given at a dose of 10 mg/kg per week for 2 weeks, has moderate activity against prostate cancer growth. This is in agreement with the previous study (31). Our present study shows that bevacizumab, when given together with 5-FU, was far more effective than monotherapy in treatment of prostate cancer. Our data, coupled with previous reports (20, 31, 39), suggest that in prostate cancer, targeting different pathways using both biological and cytotoxic compounds is needed. The safety and efficacy of bevacizumab/docetaxel/estramustine combination for hormone-refractory prostate cancer have been reported (reviewed in ref. 21). In the present study, we observe that docetaxel does not enhance the antitumor activity of bevacizumab. Experiments are under way to determine why this combination fails.

Dysregulation of cell cycle control mechanism is an important carcinogenic mechanism. The p16INK4a/cyclin D1/pRb pathway is a major regulator of the cell cycle (reviewed in ref. 40). Whereas bevacizumab/5-FU regulates the expression of several cell cycle–related components, including up-regulation of p21Waf1/Cip1, p16INK4a, and p27Kip1 and inhibition of pRb phosphorylation (P < 0.01; Fig. 4), such synergistic effects are not observed when 5-FU or bevacizumab was used as monotherapy, suggesting that
bevacizumab/5-FU also inhibits cell cycle progression. In addition, bevacizumab/5-FU may also block cell cycle progression by inhibiting the Akt activity and downstream effectors of mTOR, p70S6 kinase, and 4E-BP1. Inactivation of mTOR pathway may lead to decreased translation of mRNAs encoding positive regulators of cell cycle progression, such as cyclin D1, and to increased translation of negative regulators such as p27Kip1, as previously reported (11, 41).

Our data show that addition of bevacizumab to 5-FU-based chemotherapy potently inhibits the Akt/mTOR pathway. This observation has clinical implications because the growth and proliferation of the tumors with Akt activation and/or PTEN loss display enhanced sensitivity to rapamycin and analogues (42). These suggest that inhibition of Akt/mTOR activity by bevacizumab/5-FU could significantly contribute to treatment of prostate cancer. Apart from rapamycin and its analogues, CCI-779 and RAD001, no chemotherapeutic drugs used in treatment of prostate cancer effectively inhibit mTOR and its downstream targets. To enhance the antitumor effect of bevacizumab/5-FU, it is also worth considering to include mTOR inhibitors, such as CCI-779 and RAD001 (32, 43–45), to bevacizumab/5-FU regimen especially for patients with nonfunctional PTEN tumors or patients with metastasis and hormone-refractory tumors. Although our preclinical data hold much promise, clinical trials are needed to determine whether bevacizumab/5-FU is as effective as docetaxel/5-FU (reviewed in ref. 6) or docetaxel/prednisone (reviewed in ref. 6).

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