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High Efficacy of Docetaxel with and without Androgen Deprivation and Estramustine in Preclinical Models of Advanced Prostate Cancer

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Abstract. *Background: To assess the activity of docetaxel in combination with hormonal therapy in preclinical models of prostate cancer. Materials and Methods: Since prostate cancer has a predilection for the bone, we assessed the antitumor activity of docetaxel in in vivo models of both bone metastasis and localized prostate cancer, using MDA PCa 2b and PC3 cells in SCID mice. Results: Dramatic antitumor efficacy was observed regardless of whether the tumor cells were implanted in the prostate or in the bone. Antitumor activity was also evident in both osteolytic and osteoblastic lesions. Reasoning that docetaxel efficacy might be enhanced if it were to be used earlier in the course of the disease, we studied the sequence of docetaxel and androgen ablation (part of standard treatment for early-stage prostate cancer) in the MDA PCa 2b xenograft model. The activity was similar whether docetaxel and androgen ablation were used alone, simultaneously, or in sequence, indicating a lack of synergism or antagonism. Finally, we studied the combination of docetaxel and estramustine on androgen-sensitive and androgen-independent cell lines in vitro and in vivo. Estramustine did not increase the activity of docetaxel in these models. Conclusion: These results provide a strong preclinical rationale for the clinical development of docetaxel for the treatment of both locally advanced and disseminated prostate cancer.*

Abbreviations: PSA, prostate-specific antigen; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; SCID, severe combined immunodeficiency; SE, standard error.

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Localized prostate cancer has a favorable prognosis after definitive local therapy, but the prospect of cure diminishes significantly if the cancer escapes beyond the confines of the gland. Androgen withdrawal is currently the treatment of choice in patients with advanced prostate cancer. Most patients initially respond to hormone therapy, but all patients eventually experience relapse with androgen-independent disease. Chemotherapy has recently emerged as a therapeutic option for advanced prostate cancer (1). However, no survival benefit of this treatment has been reported to date, with randomized studies having suggested only a palliative benefit (1,2). Recently, docetaxel has emerged as one of the most promising new drugs for advanced prostate cancer (3), particularly when combined with estramustine. Phase II studies have shown a biological response (based on serum PSA decrease) in 59–88% of patients and a recent randomized phase II trial suggested both a clinical benefit and a better survival rate of the docetaxel-estramustine over the mitoxantrone-prednisone regimen (4). Results from two phase III trials comparing docetaxel-based regimens with mitoxantrone plus steroids are currently awaited.

However, only limited data are available on the activity of docetaxel in animal models of prostate cancer (5,6). One of the limitations to developing new therapy strategies for prostate cancer has been the lack of available model systems (5,6). The University of Texas M. D. Anderson Cancer Center cell culture program has devoted intense efforts to the *in vitro* growth of primary and metastatic prostate cancer. These ongoing efforts have resulted in the establishment of two human prostate cancer cell lines derived from a single bone metastasis (the MDA PCa 2a and MDA PCa 2b lines) (7,8). These new cell lines are the first available derived from a bone metastasis of an androgen-independent prostate adenocarcinoma that grow both *in vitro* and *in vivo* and have retained PSA expression and androgen sensitivity. Mouse models involving subcutaneous,

intraosseous and orthotopic (intraprostatic) xenografts of these cell lines are available and have been characterized (9,10). Moreover, only a few preclinical and clinical studies have assessed the role of the sequence of chemotherapy and androgen deprivation in prostate cancer. Since chemotherapy (with agents such as docetaxel) is currently under development for the treatment of earlier-stage prostate cancer (including locally advanced prostate cancer, biological relapse after prostatectomy and hormone-sensitive metastatic disease), this issue is now of major importance.

Here we conducted a preclinical study of docetaxel in mouse models of advanced prostate cancer. The aims were: first, to assess the anticancer activity when tumors are growing in bone (as compared with prostate); second, to study the effectiveness of different schedules of docetaxel administration and surgical castration; and, third, to evaluate the efficacy of combining estramustine with docetaxel.

Materials and Methods

Cell lines. MDA PCA 2b cells (7) were routinely propagated in BRFF-HPC1 medium (AthenaES, Baltimore, MD, USA) with 20% FBS (Gibco BRL, Gaithersburg, MD, USA). LNCaP and PC3 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 or DMEM (Gibco BRL) supplemented with 10% FBS.

In vivo studies. Six- to 8-week-old male SCID mice (Charles River Laboratory, Wilmington, MA, USA) were used for the tumorigenicity assays. The mice were housed under conditions of constant humidity and temperature, with 12-hour light/dark cycles. The mice were allowed *ad libitum* access to standard mouse feed and water and were monitored daily. The animals were anesthetized before all surgical procedures, usually with intramuscular injections of ketamine 100 mg/kg plus acepromazine 2.5 mg/kg.

Subcutaneous tumors were produced by injecting the mice with 4 x 10^6 MDA PCa 2b cells in 100 μl under the skin in the area of the right axilla. Tumor development was documented by caliper measurements made twice weekly, and tumor volume was calculated as length x width x height x 0.5236 (the formula of an ellipsoid).

Intrabone injections. We used an intrabone injection model of bone metastasis (9) in which 0.5 x 10^6 of MDA PCa 2b or PC3 cells were diluted in 3 ml of growth medium and this cell suspension was injected into the right femur of male SCID mice. Mice injected with the PCa 2b cells were then monitored bi-weekly for PSA serum levels; PSA was not monitored in mice injected with PC3 cells because PC3 cells do not produce PSA. Radiographs of the bones that had been injected were obtained twice a month and again before the mice were killed. The animals were killed 3 months after the experiment was begun (sooner if bulky tumors appeared), and the bones were removed for pathological examination as described under "Tissue samples" below.

Orthotopic model. An orthotopic model was used in which the ventral prostate of SCID mice was injected with 0.5 x 10^6 of MDA PCa 2b or PC3 cells, diluted in 3 μl of growth medium. Mice injected with MDA PCa 2b cells were monitored once a week by measuring PSA serum levels. Tumor growth was also monitored by abdominal palpation once a week.

Surgical castration was performed as previously described (7). Briefly, the animals were anesthetized and a midline scrotal incision was performed. The testes were removed and the spermatic cord was ligated 1 cm above the junction of the cord and the testis. The scrotum was subsequently closed with wound clips.

Tissue samples. After the mice had been killed, the tumors were removed, fixed in formalin, and embedded in paraffin by the Department of Veterinary Medicine of M. D. Anderson Cancer Center, USA. To examine bone tumors, the bones were dissected free of muscle, fixed in 10% buffered formalin, decalcified in 5% formic acid and then embedded in paraffin. Longitudinal 3-μm-thick sections were obtained from each sample block and stained with hematoxylin and eosin.

Serum PSA levels. Blood samples were obtained by venipuncture by means of a small incision in the main tail vein. Serum was separated from the blood and PSA was measured in the serum fraction by using a microparticle enzyme immunoassay (IMx PSA assay, Abbott Laboratories, Abbott Park, IL, USA).

Drugs. Docetaxel, kindly provided by Aventis (Somerset, NJ, USA), was dissolved in ethanol and polysorbate 80 was added. The final dilution was made just before injection with 5% dextrose (5/5/90 vol/vol/vol). Each mouse was given an *i.v.* dose of 10 mg/kg body weight estramustine phosphate. Estramustine was kindly provided by Pharmacia (Peapack, NJ, USA).

Statistical analysis. Numerical data were expressed as means ± SE. Statistical differences between means for the different groups were evaluated with Sigma Plot 4 one-way analysis of variance and Tukey's mean separation test, with the level of significance set at $p<0.05$.

Table I. *Serum PSA blood levels (mean values in ng/ml) in mice with intraosseous or intraprostatic MDA PCa 2b cell xenografts treated or not treated with i.v. docetaxel.*

Injection site	Measurement time			
and treatment	Baseline	Week 1	Week 2	Week 6
Femur				
Control	3.2	5.8	7.1	107.5
Docetaxel	3.2	1.0	0.4	2.5
Prostate				
Control	2.0	7.1	10.6	151.1
Docetaxel	2.5	0.9	0.4	1.4

Results

Determination of docetaxel dose in SCID mice. Until now, docetaxel has been assessed mostly in nude mice and in rats. Because our models of advanced prostate cancer were developed in SCID mice (9,10), we performed a pilot study to determine the doses of docetaxel to be used. Eight SCID

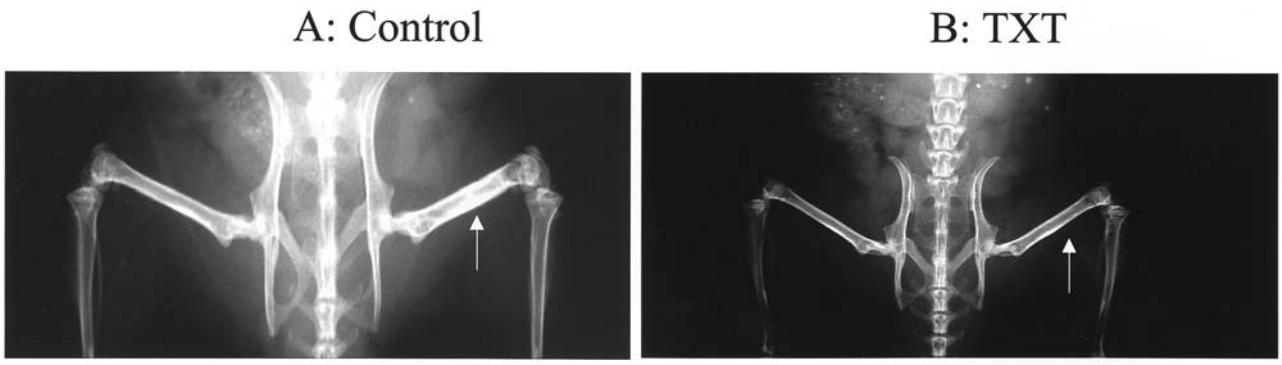

Figure 1. *Antitumor activity of docetaxel in a model of osteoblastic bone metastases from prostate cancer. Mice were injected with MDA PCa 2b cells in the distal extremity of the femur, followed by X-ray analysis of the injected limbs. Osteoblastic lesions (arrow) appeared in week 6 in controls (A) and were prevented in treated animals (B).*

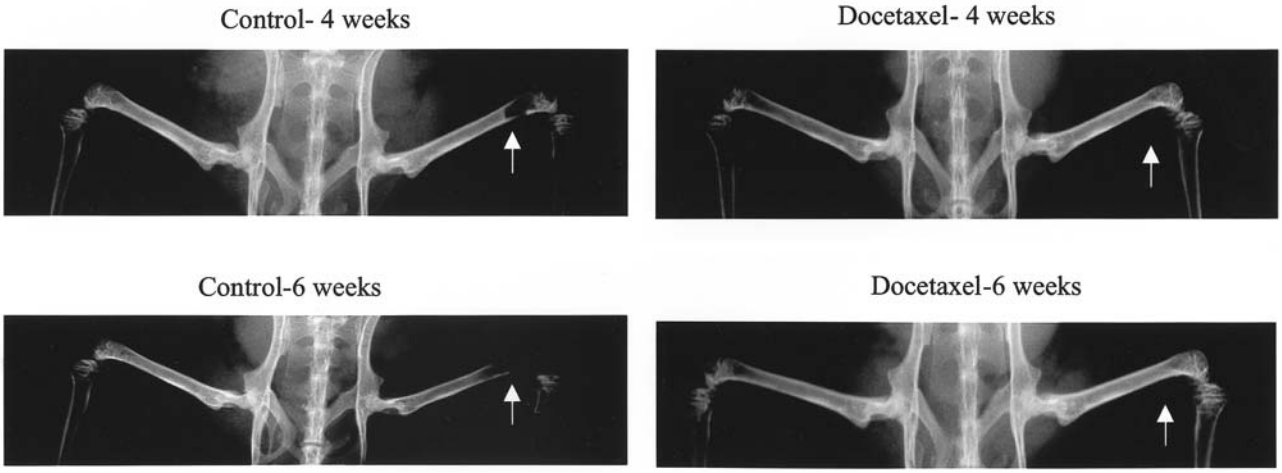

Figure 2. *Antitumor activity of docetaxel in a model of osteolytic bone metastases from prostate cancer. Mice were injected with PC3 cells in the distal extremity of the femur and followed by X-ray for 6 weeks. Lytic lesions (arrow) appeared in week 4 in controls and were prevented in treated animals.*

mice were injected with 10^6 PC3 cells subcutaneously. After the tumors had reached 400 mm^3, the mice were randomized to receive docetaxel at one of three doses: 15 mg/kg x 3, 22 mg/kg x 3, or 0 mg/kg x3 (vehicle-only control). Efficacy and toxicity were assessed 3 times a week by measuring tumor volume and body weight. The mean weight loss was 21% for mice in the 15 mg/kg group and 19% for the 22 mg/kg group. However, the mean weight loss in the control mice was 13%, indicating that most of the weight loss had been due to tumor growth rather than drug toxicity. PC3 tumors in all 3 control mice progressed rapidly. In contrast, 4 of the 5 mice given docetaxel showed a decrease in tumor volume after therapy. Another group of 8 mice bearing either a subcutaneous MDA PCa 2b tumor (4 x 10^6 cells) (n=3) or no tumor were treated under the same conditions and received docetaxel at a dose of 10 mg/kg x3, 15 mg/kg x3, or 22 mg/kg x3. The mean weight loss was 10%, 15% and 15%, respectively. All 3 mice bearing an MDA PCa 2b tumor showed a partial response to docetaxel at 15 mg/kg. On the basis of these results, we decided to use a docetaxel dose of 15 mg/kg x 3, which is the same dose as was previously recommended for nude mice (11).

Assessment of docetaxel activity in bone lesions from prostate cancer. Prostate cancer typically metastasizes to the bone, where it causes osteopetrotic ("osteoblastic") lesions. Since bone has been considered a sanctuary site for prostate cancer, we sought to determine whether a chemotherapeutic agent would have equivalent antitumor activity against prostate cancer developing in the bone *versus* that developing in the prostate (orthotopic model).

MDA PCa 2b cells were suspended in serum-free medium and injected into either the prostate or the femur of 26 SCID mice. After 4 weeks, the mice were randomized to receive either 3 doses of docetaxel at 15 mg/kg or no therapy (surveillance group). Serum PSA levels were assessed before

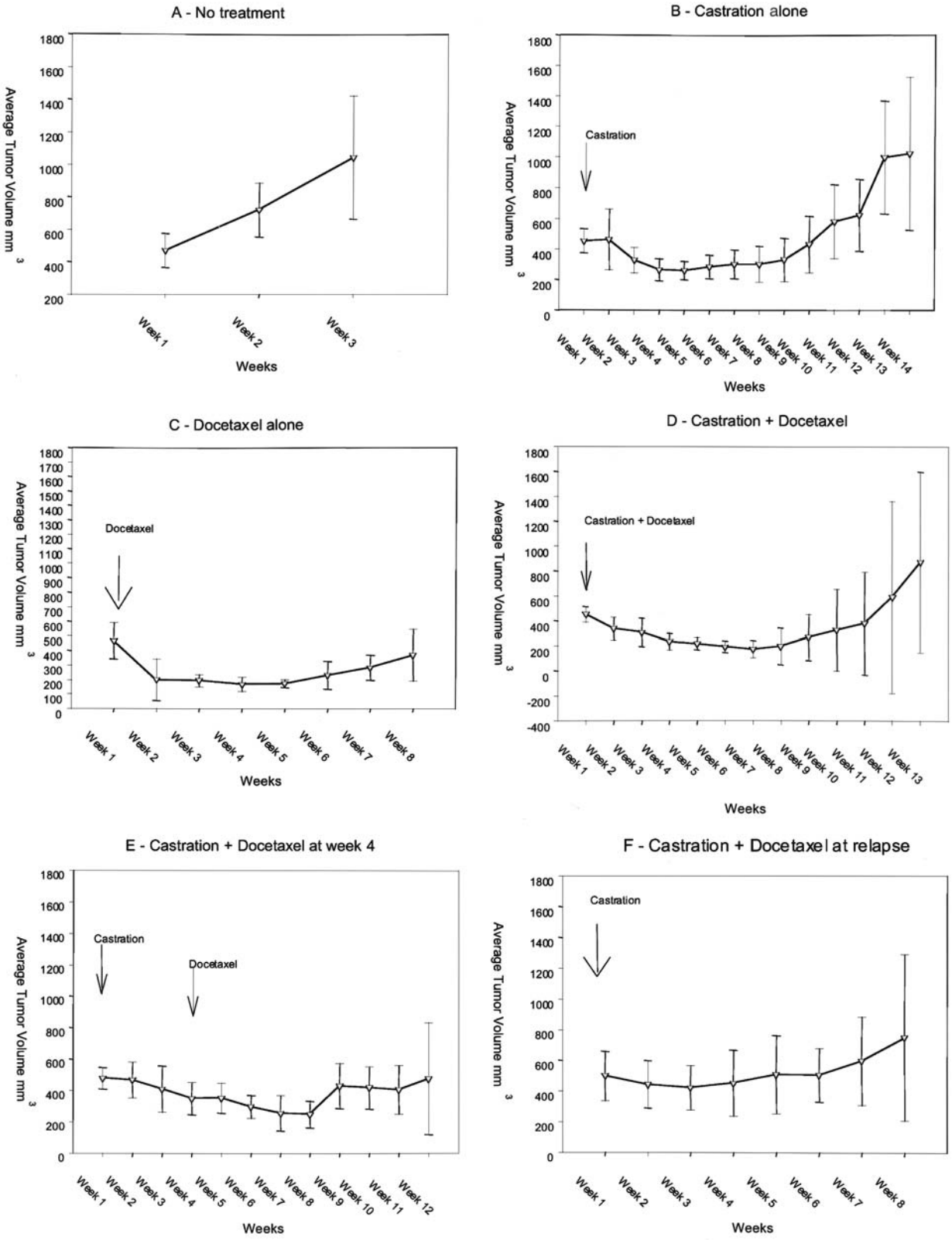

Figure 3. *Antitumor activity resulting from different sequences of docetaxel and androgen ablation in the MDA PCa 2b xenograft model. A, controls (untreated animals); B, castrated animals; C, docetaxel alone (no castration); D, docetaxel used synchronously with castration; E, docetaxel begun 4 weeks after castration; F, castration followed by docetaxel in case of progression.*

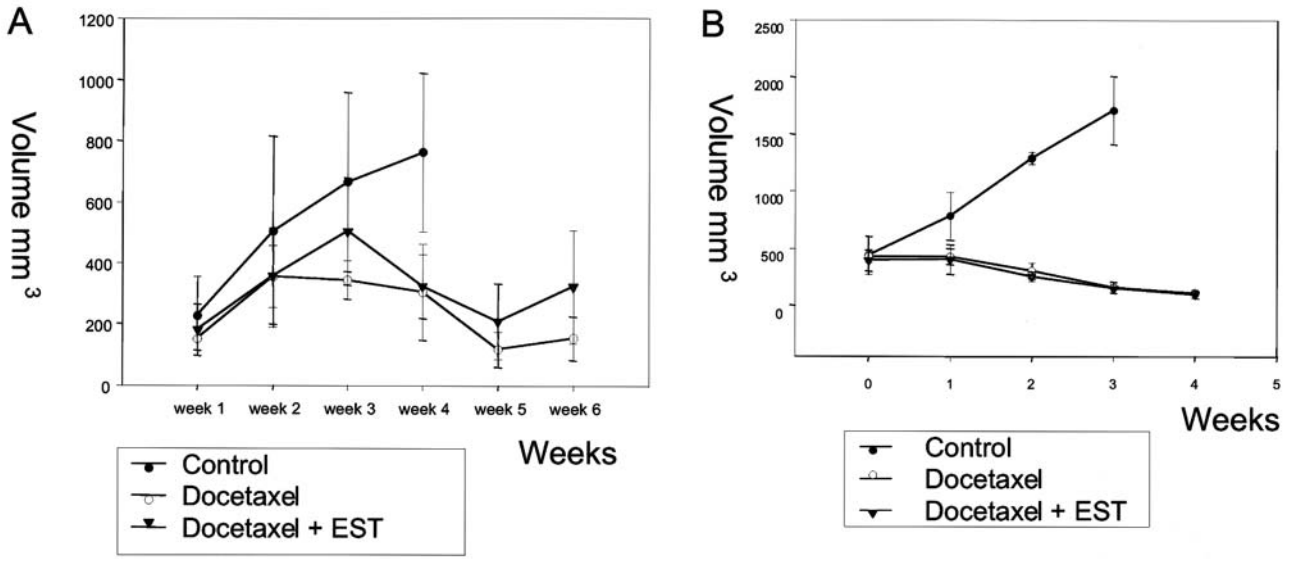

Figure 4. *Antitumor activity of docetaxel with or without estramustine (EST) in xenograft (s.c.) models of prostate cancer. A, MDA PCa 2b tumors; B, PC3 tumors.*

Table II. *In vitro viability of three prostate cancer cell lines treated with docetaxel and estramustine phosphate.*

	IC_{50} (nM)	
Cell line	Docetaxel	Docetaxel + Estramustine
PC3	60	60
LNCaP	6	15
MDA Pca 2b	30	60

therapy and biweekly thereafter. Mice injected in the femur (n=13) were checked every 2 weeks by X-ray and mice injected in the prostate (n=13) were checked once a week for tumor growth. Docetaxel rapidly and substantially reduced serum PSA levels beginning the first week after treatment (Table I). The decrease in PSA levels was similar regardless of whether the tumor cells were in the bone or the prostate. Three weeks after the docetaxel injections, the PSA level had decreased by a mean of 98% (for mice with bone tumors) and 99% (for mice with prostate tumors). Docetaxel prevented the formation of osteoblastic lesions following injection of MDA PCa 2b cells into the bone (Figure 1). Good tolerance in SCID mice of the 15 mg/kg x 3 docetaxel schedule was confirmed.

In a similar experiment, PC3 cells were also injected into the femur (n =10) or the prostate (n =10) of SCID mice. Docetaxel (at 15 mg/kg, x 3) injected 2 weeks after tumor cell inoculation completely prevented the formation of bone lesions (Figure 2) and inhibited tumor growth into the prostate. (PSA levels could not be evaluated because PC3 cells do not produce PSA.).

Does androgen ablation modify the antitumor activity of docetaxel in prostate cancer? In human prostate cancer, docetaxel is used in the hormone-refractory stage of the disease. It is expected that docetaxel would have greater activity if it were used earlier in the disease, however this possibility has yet to be evaluated. Therefore, the combination of docetaxel with androgen ablation (which is part of the standard treatment of earlier-stage disease) may be more effective than docetaxel alone. We thus attempted to determine, in preclinical models, whether docetaxel and castration, used in a synchronous or a metachronous schedule, had any advantage over docetaxel alone. Fifty mice were injected with 4 x 10^6 MDA PCa 2b cells subcutaneously. When the tumors reached 500 mm^3, the mice were randomly assigned to undergo various combinations of docetaxel and castration. The endpoint was the time for the tumor volume to reach 1000 mm^3. The results are summarized in Figure 3. Tumors in the untreated control group grew rapidly, reaching 1000 mm^3 in 3 weeks. In castrated mice (group B), the tumors shrank after castration and then grew after a delay of roughly 10 weeks, reaching 1000 mm^3 in approximatelly 13 weeks. Serum PSA levels tended to follow the response and tumor progression (data not shown). The efficacy of docetaxel used as a single agent was confirmed in this model (group C) and these results were not affected by the addition of simultaneous castration with the docetaxel (group D). When docetaxel was started 4 weeks after castration (group E), relapses were slightly delayed and the mean tumor volume at 12 weeks was 500 mm^3 (*versus* 625 mm^3 in castrated mice, with or without simultaneous docetaxel). However, this apparent difference was not statistically

significant, and it cannot be concluded that separate administration of castration and docetaxel resulted in higher activity than simultaneous administration.

Is docetaxel synergistic with estramustine in prostate cancer? Whether estramustine has a role in the clinical treatment of prostate cancer remains questionable. However, the possibility exists that estramustine may be more effective when used in combination with docetaxel. To assess whether the combination of estramustine is synergystic with docetaxel in prostate cancer, we used three human prostate cancer cell lines for *in vitro* cytotoxicity experiments: PC3, which is androgen-independent, and LNCaP and MDA PCa 2b, which are both sensitive to androgen deprivation. *In vitro*, the concentrations of docetaxel required to reduce human cancer cell survival by 50% ranged from 4 to 35 ng/ml (12). Therefore, doses of docetaxel ranging from 10^{-4} to 60 nM (which covers the range of active doses *in vitro*) were used, with and without 100 nM estramustine. Estramustine did not reduce the IC_{50} in any of the three cell lines tested (Table II).

To test this combination *in vivo*, 21 mice were given *s.c.* injections of MDA PCa 2b cells. When tumors reached a volume of 200 mm^3, the animals were randomized to receive 3 doses of docetaxel 15 mg/kg, with or without estramustine (10 mg/kg body weight) or no therapy (control group). At 5 weeks after treatment, the tumor volume had reached 1200 mm^3 in the control group, whereas the mean tumor volume in the docetaxel group was 100 mm^3 and that in the docetaxel + estramustine group was 200 mm^3 (Figure 4).

In a similar experiment in which 15 mice were given *s.c.* injections of PC3 cells, tumors grew rapidly in the control group, but a complete response was obtained in 6 weeks in mice treated with docetaxel, regardless of whether they also received estramustine (Figure 4). The kinetics of tumor shrinkage were the same in both drug-treatment groups.

We concluded that estramustine and docetaxel do not have synergistic effects in these models of prostate cancer.

Discussion

Docetaxel has shown promise for the treatment of advanced prostate cancer. This study showed that docetaxel had antitumor activity *in vivo* when prostate cancer cells were localized either in the bone or the prostate. Docetaxel prevented the development of the lytic lesions typically induced by the very aggressive, hormone-insensitive, PC3 cell line. We also showed that docetaxel had antitumor activity in the MDA PCa 2b model independently of androgen deprivation. Finally, no evidence of any synergistic activity with estramustine was found *in vitro* or *in vivo* in three models of prostate cancer.

Until recently, few models were available to assess preclinical drug activity in bone metastases from prostate cancer. To our knowledge, the only *in vivo* data on docetaxel in prostate cancer published so far were obtained in Copenhagen rats injected subcutaneously with the Mat LyLu cell line (6). In humans, clinical responses of bone metastases have been considered difficult to assess, and clinical assessment of new drugs is mostly based on decreases in serum PSA levels and on quality-of-life measurements (13). Moreover, chemotherapy was thought to be less efficient for prostate cancer than for other neoplasms because of its high affinity for the bone, which has been regarded as a sanctuary site. The recent availability of orthotopic models and of models of both osteoblastic and osteolytic lesions induced by prostate cancer prompted us to study the *in vivo* activity of docetaxel. Our findings demonstrated that docetaxel had substantial antitumor efficacy in both androgen-dependent and androgen-independent models of prostate cancer. Moreover, antitumor activity was observed in both osteolytic and osteoblatic lesions, which has not previously been shown for any chemotherapeutic drug, either in humans or in preclinical models.

Docetaxel arrests cells in the G_2/M-phase of the cell cycle and induces apoptosis. Docetaxel probably induces these effects through two mechanisms: it inhibits microtubule depolymerization by binding to β-tubulin (14) and it also induces bcl-2 phosphorylation, which abrogates its anti-apoptotic function, resulting in cell death (14,15). Because androgen ablation and docetaxel do not have similar mechanisms of action, and because these treatments are likely to be used in combination in the clinical setting, we thought it important to study whether the sequence of application of these two treatments would affect their activity. We found no difference in efficacy when docetaxel and androgen ablation were given sequentially or simultaneously. Recently, Muenchen *et al.* showed that the pathway for docetaxel-induced apoptosis differs between androgen-sensitive and androgen-independent prostate cancer cells: caspase-3 and caspase-7 are cleaved in the former case, whereas caspase-8 is cleaved in the latter case (16). Taken together, these findings suggest that docetaxel might be useful not only for hormone-refractory disease but also for hormone-sensitive and hormone-naive disease.

The role of estramustine with docetaxel in the clinic remains questionable. Estramustine is thought to exert antitumor activity by interfering with microtubule function and potentially by blocking the phosphorylation of mutated androgen receptors (17). Although a synergistic effect has been reported for many chemotherapeutic agents, the efficacy of estramustine as a single agent is notoriously low. Moreover, a randomized trial recently reported no significant survival benefit for the combination of vinblastine and estramustine as compared to vinblastine alone, although estramustine markedly increased the toxicity and probably

the cost as well (18). In humans, evidence now exists that single-agent docetaxel is active in prostate cancer and results from an international phase III trial using this regimen are awaited soon. The preliminary results of a randomized phase II trial suggested that estramustine may enhance the activity of paclitaxel (19). Although the findings from one preclinical study suggested synergism between docetaxel and estramustine (6), our results, obtained with both *in vivo* and *in vitro* models employing three prostate cancer cell lines (including both androgen-sensitive and androgen-independent cell lines), did not indicate synergistic activity. Therefore, the question of whether estramustine contributes to the efficacy of the docetaxel-estramustine combination still requires further study.

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