Taxol and Estramustine-induced Modulation of Human Prostate Cancer Cell Apoptosis via Alteration in bcl-x$_L$ and bak Expression$^1$

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ABSTRACT
bcl-x$_L$ is an antiapoptotic protein that shares sequence homology with bcl-2 and seems to convey chemoresistance in many human tumor cell lines. bcl-x$_L$ protein is expressed at a 3-fold higher level in PC-3 cells than it is in LNCaP cells. Taxol causes apoptosis in both these cell lines, as measured by the formation of DNA ladders and by the observation of typical cellular morphological changes (chromatin condensation and nuclear fragmentation) after 4',6-diamidino-2-phenylindole staining. Overexpression of bcl-2 in LNCaP cells did not prevent Taxol-induced apoptosis. Treatment of LNCaP cells with 10 nM Taxol led, after 24 h, to relatively specific and almost total down-regulation of bcl-x$_L$ protein in the absence of alteration of bax, bak, or bcl-2 levels. This change was paralleled by a similar decrease in the level of bcl-x$_L$ mRNA, as demonstrated by reverse transcription-PCR. The level of glyceraldehyde-3-phosphate dehydrogenase mRNA was not changed. In PC-3 cells, 48 h were required for both maximal bcl-x$_L$ protein down-regulation and cellular apoptosis. In contrast, treatment of LNCaP cells with estramustine induced apoptosis, but this was not associated with any change in the intracellular level of bcl-x$_L$ or bax protein. Instead, relatively specific 2-fold up-regulation of the proapoptotic protein bak was observed. In PC-3 cells, cellular apoptosis induced by estramustine was bak independent. These results augment our understanding of the importance of bcl-x$_L$ in prostate cancer and suggest that appropriate manipulation of cytotoxic chemotherapeutic agents may favorably alter the balance between pro- and antiapoptotic proteins in this tumor.

INTRODUCTION
Several pathways have been recently described that regulate programmed cell death (apoptosis) during development and tumorigenesis. Cells from a wide variety of human malignancies have a decreased ability to undergo apoptosis in response to at least some physiological stimuli (1). Several genes that are critical in the regulation of apoptosis have recently been defined (2). One of these genes, first described in immune cells and growth factor-dependent neurons, is the bcl-2 gene. Its homologues have also recently been characterized (3–6). bcl-2 family members have been shown to contribute to tumorigenesis by inhibiting apoptotic signals that are generated during the activation of certain oncogenes, such as c-myc or the p53 tumor suppressor gene (7). Moreover, bcl-2 and the related gene bcl-x$_L$ can provide signals that rescue tumor cells from apoptosis that has been induced by chemotherapeutic drugs, thus implicating these survival proteins in the resistance of cancer cells to chemotherapy (7). Induction of apoptosis by antitumor agents may require an increase in wild-type p53 expression and may also be mediated through a decrease in bcl-2 and bcl-x$_L$ and/or an increase in a related proapoptotic gene, bak (6).

Chemotherapeutic agents may induce tumor cell death by causing damage that induces the cell to commit suicide (2, 8). Taxol, a microtubule stabilizer, has been shown to affect numerous microtubule-regulated functions, resulting in inhibition of cell proliferation and mitotic arrest (9, 10), induction of microtubule bundling (11), formation of abnormal mitotic spindle asters (12–14), and inhibition of transmission of transmembrane mitogenic signals (15). Clinically, Taxol is an active clinical agent in the treatment of many tumor types, including carcinoma of the breast and ovary (16). Estramustine, also an active agent in hormone-refractory prostate cancer, is thought to be an inhibitor of microtubule polymerization (17). The combination of Taxol and estramustine is currently under clinical investigation for this disease, and the initial results are encouraging (18),$^3$

In studies in tissue culture, the induction of apoptosis by Taxol in human PC-3 prostate carcinoma cells was associated with internucleosomal DNA fragmentation (19). Furthermore, Taxol has recently been suggested to induce phosphorylation of the bcl-2 protein and also to cause LNCaP cells to undergo apoptosis (20, 21). However, our initial experiments demonstrated that the expression of bcl-x$_L$ in LNCaP and PC-3 prostate cancer cells was much greater than that of bcl-2. Thus, we determined whether Taxol-induced apoptosis could also be mediated through alteration in the levels of bcl-x$_L$ or other members of this family, such as the proapoptotic proteins bax and

Received 2/7/97; revised 4/18/97; accepted 6/6/97.

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$^1$This work was supported by a CapCure/David Koch Foundation Grant to Q-Y. L. and C. A. S.

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$^3$D. Petrylak, personal communication.
Induction of Apoptosis by Taxol and Estramustine

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Fig. 1 A. immunoblot analysis of bak, bax, and bcl-xL proteins in human prostate cancer cell lines. Protein samples (40 μg total protein/ lane) were derived from LNCaP (LN) and PC-3 cells and subjected to Western blotting with a polyclonal antibody as described in the text. B. Northern blot analysis of bcl-x and GAPDH expression in LNCaP (LN) and PC-3 cells, performed as described in the text. The probe for bcl-x was a 700-bp coding region cDNA fragment generated by RT-PCR.

In addition, because the Taxol/estramustine combination may be clinically active, we have performed similar experiments with estramustine. We confirm that Taxol induced concentration-dependent apoptosis in both the LNCaP and PC-3 lines. Western blot analysis of bcl-xL protein levels showed that Taxol-induced apoptosis is mediated through relatively specific down-regulation of bcl-xL. Analysis of bcl-xL mRNA levels in LNCaP cells by RT-PCR revealed that the message was down-regulated after a 4-h treatment with drug and was not detected after 24 h. In contrast, estramustine treatment of LNCaP cells caused relatively specific up-regulation of bak protein expression and apoptosis without a change in the level of bcl-xL or bax protein expression.

**MATERIALS AND METHODS**

**Cells.** LNCaP and PC-3 prostate cancer cells were purchased from American Tissue Type Collection (Rockville, MD) and cultured in RPMI 1640 supplemented with 10% FCS in 95% air/5% CO₂. The bcl-2-transformed LNCaP cells (Ref. 25; generously donated by R. Buttyan and A. Raffo, Columbia University, New York, NY) were cultured in identical media with the addition of 0.3 mg/ml G418. Cells in complete media were treated with different concentrations of Taxol (from Bristol-Myers/Squibb) or estramustine (Kabi, Helsingborg, Sweden; dissolved in DMSO) for the times as indicated in “Results.” The

cells were harvested, and the proteins and total RNA were extracted as described below.

**Demonstration of Internucleosomal DNA Cleavage.** DNA fragmentation assays were performed as described with modification (26). Briefly, 2 × 10⁶ LNCaP cells, treated with 1–100 nM Taxol for the indicated times, were lysed in 1% NP40, 20 mM EDTA, and 50 mM Tris-Cl (pH 7.5; 100 μl/10⁶ cells). After centrifugation for 5 min at 15,000 × g, the supernatant was collected, and the extraction was repeated with an additional 50 μl/10⁶ cells of lysis buffer. The supernatants were brought to a 1% SDS concentration and treated with RNase A (final concentration, 200 μg/ml) at 56°C for 1 h followed by digestion with proteinase K (final concentration, 2.5 mg/ml) for 2 h at 56°C. The DNA was then precipitated with 2.5 volumes of ethanol and dissolved in 10 mM Tris (pH 8)-1 mM EDTA buffer (pH 7.4). Equal amounts of DNA, as measured by UV absorbance at 260 nm, were electrophoresed on 1.2% agarose gels containing 0.5 mg/ml ethidium bromide and visualized by UV transillumination.

**Detection of Apoptotic Nuclei by DAPI Staining.** Morphological changes characteristic of apoptosis were determined by staining cell nuclei with DAPI. Briefly, cells were plated onto poly-d-lysine-coated tissue culture chamber slides (Nunc, Inc., Naperville, IL) and incubated overnight. The cells were treated with Taxol (20 nM) or estramustine (10 μM) for 24 h and then washed once with PBS. The cells were then fixed with 90% ethanol/5% acetic acid for 1 h at room temperature, washed twice with PBS, and treated with a 1.5 mg/ml solution of DAPI in PBS for 30 min at room temperature. The slides were washed twice with PBS, mounted, and photographed using a Nikon phase-fluorescence microscope.

**RNA Isolation and Northern Blot Analysis.** Total RNA was isolated from 5 × 10⁷ cells using the guanidine isothiocyanate method as described previously (27). Twenty μg of total RNA were electrophoresed on a 1% denaturing agarose gel containing formaldehyde, transferred to a Duralose-UV filter (Stratagene, La Jolla, CA), and covalently bound by UV irradiation. A radiolabeled bcl-xL coding region cDNA fragment generated by RT-PCR (see below) and labeled by the Random Primers DNA Labeling System kit (Life Technologies, Inc.) was hybridized to the filter-bound RNA in 50% formamide, 5× saline-sodium phosphate-EDTA, 5× Denhardt’s solution, 0.1% SDS, and 10 mg/ml yeast tRNA overnight at 42°C. The filter was washed twice for 5 min at room temperature in 2× SSC and 0.1% SDS, once for 5 min at 42°C in 1× SSC and 0.1% SDS, and, finally, twice for 10 min in 0.1× SSC and 0.1% SDS at 55°C. The filter was exposed to Kodak X-ray film with intensifying screens for 24–48 h at −70°C and developed.

**RT-PCR.** The first-strand cDNA was synthesized from 2 μg of total RNA isolated from cells treated with Taxol in a 20-μl reaction mixture containing 4 μl of 5× RT reaction buffer, 10 units of RNasein (Promega, Madison, WI), 200 μM deoxynucleotide triphosphate, 40 pm oligodeoxynucleotidyl acid primer, and 20 units of Moloney murine reverse transcriptase (Promega). The mixture was incubated at 42°C for 1 h and then incubated at 37°C for 30 min. The unhybridized RNA was then digested with 10 units of RNase H at 37°C for 10 min. The RT products were diluted to 200 μl with 10 mM Tris (pH 8)-1 mM EDTA buffer, and 4 μl of the diluted RT products were sub-

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4 The abbreviations used are: RT-PCR, reverse transcription-PCR; mAb, monoclonal antibody; DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ECL, enhanced chemiluminescence.
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Fig. 2 DNA laddering in LNCaP and PC-3 cells after treatment with Taxol or estramustine. A. LNCaP cells (2 x 10⁶) were treated with 100 nM Taxol for the stated times. m, 123-bp DNA ladder marker (Life Technologies, Inc.); ln, untreated LNCaP cells. DNA was isolated as described in the text, electrophoresed on a 1.2% agarose gel, and stained with 0.5 mg/ml ethidium bromide. B. LNCaP cells were treated with 1, 5, 10, and 20 nM Taxol for 24 h. DNA was isolated as described in the text and visualized as described in A. C. PC-3 cells were treated with 10 nM Taxol for 1, 2, and 3 days. DNA was isolated as described in the text and visualized as described in A.

Fig. 3 Apoptosis in LNCaP cells as visualized by DAPI staining after Taxol or estramustine treatment. Cells were grown in poly-D-lysine-coated tissue culture chamber slides, fixed, and stained with 1.5 mg/ml of DAPI for 30 min. A, untreated LNCaP cells. B, LNCaP cells were treated with 10 nM Taxol for 24 h, by which time most cells clearly undergo apoptosis as detected by changes in nuclear morphology. C, LNCaP cells were treated 1 µM estramustine for 24 h. Nuclear fragmentation and chromatin condensation are clearly visible in most cells.

Western Blot Analysis. Approximately 10⁶ cells were lysed in 100 µl of ice-cold radioimmunoprecipitation assay buffer [50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40, and 0.5% sodium deoxycholate] with freshly added 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1 µg/ml aprotinin, mixed gently with a pipette, and incubated on ice for 30 min. Cell debris was removed by centrifugation at 15,000 x g for 10 min at 4°C. Protein concentrations were determined using the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA). Aliquots of cell extracts containing 25 µg of protein were resolved by 12% SDS-PAGE (28) and transferred to Hybond ECL filter paper (Amer sham, Arlington Heights, IL). Filters were incubated at 25°C for 1 h in Blotto A [5% nonfat milk powder in TBS:10 mM Tris-HCl (pH 8.0); 150 mM NaCl] plus 0.05% Tween 20 and then incubated at 25°C for 1 h in Blotto A containing a 1:200 dilution of either rabbit anti-bcl-x, anti-bax, or anti-bak poly-
Induction of Apoptosis

The expression of apoptosis-modulating genes was analyzed in LNCaP and PC-3 cells using Western and Northern blotting. The Western blot results are shown in Fig. 4A. The blots contained equivalent amounts of proteins in each lane and were probed with anti-bcl-x, anti-bax, and anti-bak antibodies. The apoptosis-inducing gene bak was expressed at a lower level in both cell lines, whereas bax was expressed, as assessed by laser scanning densitometry, at an approximately 3-fold higher level than bak. bcl-x₁ expression was approximately 3-fold higher in PC-3 cells than in LNCaP cells. In contrast, bcl-2 protein expression seemed to be minimal in both of these cell lines.

The relatively high expression of bcl-x₁ in each cell line was confirmed by Northern blot analysis of total RNA isolated from LNCaP and PC-3 cells using a bcl-x₁ fragment derived from RT-PCR amplification of bcl-x₁ mRNA as probe. The approximately 3.3-kb bcl-x₁ transcript was detected in both cell lines. bcl-x₁ mRNA was found at approximately a 3-fold higher level in PC-3 compared to that of LNCaP cells (Fig. 1B). In contrast, neither cell line expressed bcl-x₂ mRNA, as determined by RT-PCR amplification of total RNA using specific primers (data not shown).

The Apoptosis of Prostate Cancer Cells in Response to Taxol Is Concentration- and Time-dependent. To determine whether Taxol-induced LNCaP death demonstrates the characteristic DNA laddering of apoptosis, we examined genomic DNA after treatment with 100 nm Taxol. As shown in Fig. 2A, internucleosomal fragmentation of the DNA was observed in the cells after treatment for 24 h. We then examined the dose response to Taxol in LNCaP and PC-3 cells. The data (Fig. 2B) demonstrate that DNA laddering occurred in LNCaP cells after incubating them in 10 nm Taxol (but not at 0.5 or 1 nm) for 24 h. However, internucleosomal DNA cleavage did not occur with PC-3 cells at this Taxol concentration until after 48 h of incubation time (Fig. 2C). To demonstrate that Taxol induced cellular apoptosis, we stained the cells with the nuclear stain DAPI. As shown in Fig. 3B, chromatin condensation and nuclear fragmentation were observed in most LNCaP cells after 10 nm Taxol treatment.

Taxol-induced Apoptosis of Human Prostate Cancer Cells Is Mediated by Down-Regulation of bcl-x₁. We analyzed the changes in expression of bcl-x₁ protein in LNCaP and PC-3 cells in response to Taxol treatment. As shown in Fig. 4A, bcl-x₁ protein levels are strikingly down-regulated by incubation with 100 nm Taxol in LNCaP cells. Down-regulation was clearly observed after 6 h, and bcl-x₁ protein was almost undetectable in the cells treated with 100 nm Taxol for 24 h. We then evaluated the expression of bcl-x₁ protein in PC-3 cells under...
cells. LNCaP/bcl-2 cells were treated with 100 nM Taxol for 24 h. DNA was isolated and electrophoresed as described in the text and visualized by ethidium bromide staining. m, 123-bp DNA ladder marker. B, immunoblot analysis of bcl-2 protein in bcl-2-transformed LNCaP, parental LNCaP, and PC-3 cells. LNCaP/bcl-2 cells were treated with 100 nM Taxol for the stated times. Parental LNCaP cells were treated with 100 nM Taxol for 24 h. A total of 25 μg protein/lane were electrophoresed. Untreated LNCaP/bcl-2, parental LNCaP and PC-3 cells were used as controls. The blots were probed with an anti-bcl-2 mAb as described in the text. Phosphorylation of bcl-2 can be clearly seen after treatment of the LNCaP/bcl-2 cells with Taxol for 24 h.

**Fig. 6** DNA laddering and Western blot analysis of bcl-2-transformed LNCaP cells after Taxol treatment. A, DNA fragmentation of LNCaP/bcl-2 cells after treatment with 1, 5, 10, and 20 nM Taxol for 24 h. DNA was isolated and electrophoresed as described in the text and visualized by ethidium bromide staining. m, 123-bp DNA ladder marker. B, immunoblot analysis of bcl-2 protein in bcl-2-transformed LNCaP, parental LNCaP, and PC-3 cells. LNCaP/bcl-2 cells were treated with 100 nM Taxol for the stated times. Parental LNCaP cells were treated with 100 nM Taxol for 24 h. A total of 25 μg protein/lane were electrophoresed. Untreated LNCaP/bcl-2, parental LNCaP and PC-3 cells were used as controls. The blots were probed with an anti-bcl-2 mAb as described in the text. Phosphorylation of bcl-2 can be clearly seen after treatment of the LNCaP/bcl-2 cells with Taxol for 24 h.

identical conditions. In contrast to what was observed in the LNCaP cells, the expression level of bcl-xL remained unchanged after treatment with 100 nM Taxol for 6 h. However, after 24 h of Taxol treatment, bcl-xL protein expression began to decrease, and it was almost undetectable only after 48 h (Fig. 4B). The level of expression of other proteins related to the apoptotic process, including bcl-xS, bax, bak, and fas, was unchanged by treatment with Taxol.

To determine whether the down-regulation of bcl-xL protein induced by Taxol was associated with a decrease in bcl-xL mRNA, we next assessed the expression of bcl-xL mRNA in LNCaP cells by semiquantitative RT-PCR analysis. As shown in Fig. 5, bcl-xL message levels were clearly down-regulated after 4 h of treatment with Taxol and were undetectable after 24 h. This result correlates with down-regulation of bcl-xL protein observed by Western blotting. The level of the proapoptotic bcl-xL remained undetectable throughout the course of treatment with Taxol (data not shown).

**Overexpression of bcl-2 Does Not Protect LNCaP Cells from Taxol-induced Apoptosis.** In addition to causing cellular apoptosis (20), Taxol has recently been suggested to phosphorylate bcl-2 protein in RS1846 lymphoma cells (21) and in prostate cancer cells as well. To determine whether overexpression of bcl-2 altered the cellular response to Taxol-induced apoptosis, we investigated the response of bcl-2-transformed LNCaP cells to Taxol. Overexpression of bcl-2 in LNCaP bcl-2-transformed cells did not render these cells resistant to Taxol-induced apoptosis. As shown in Fig. 6A, DNA laddering was induced by the same conditions as those used to treat the parental LNCaP cells (10 nM Taxol for 24 h). In agreement with previous reports (20, 21), Taxol induced the presence of two bands that were recognized by the anti-bcl-2 mAb in the bcl-2-transformed LNCaP cells (Fig. 6B, 24 h). The presence of two bands has previously been interpreted as indicating the induction of phosphorylation of bcl-2 by Taxol.

To our surprise, however, we could detect little or no bcl-2 protein in parental LNCaP and PC-3 cells, in contrast to previous reports (20, 21). Our failure to detect bcl-2 protein in LNCaP and PC-3 cells in our experiment may be due to different specificities of the antibodies used, but it is more likely that the level of expression level of bcl-2 in our cell samples is just too low to be detected by our Western blotting techniques.

**Estramustine-induced Apoptosis in LNCaP Cells Is Accompanied by Up-Regulation of bak Protein Expression.** When LNCaP cells were treated with 5 and 10 μM estramustine for 24 h, clear evidence of DNA laddering (Fig. 7) and nuclear chromatin condensation (see Fig. 3C) was obtained. In contrast, when the estramustine concentration was 1 μM, 72 h were required for intranucleosomal DNA cleavage to be observed. We then performed Western blotting to determine the levels of apoptosis-related proteins subsequent to estramustine treatment. As shown in Fig. 8A, there is a time-dependent increase in bax protein expression in LNCaP cells during treatment with 10 μM estramustine. This increase (approximately 2-fold, as determined by laser scanning densitometry) is maximal at approximately 24–48 h. Neither levels of bcl-xL (Fig. 8B) nor bax (data not shown) proteins changed under these conditions. In contrast, the level of bax expression in LNCaP cells (Fig. 8C) also did not change under these conditions, although estramustine (10 μM) is still capable of inducing intranucleosomal cleavage under these conditions (data not shown).

In LNCaP cells stably transfected with bcl-2, treatment with estramustine (10 μM, 18–24 h) led to an approximately 2-fold decrease in expression of bcl-2 protein and to its partial
bcl-x protein expression has also been found in the epithelial cells of the normal prostate gland (29), where, as has been speculated, it contributes to the hormone-dependent control of programmed cell death. In a recent series (33) of 64 cases of adenocarcinoma of the prostate, all (100%) stained positively for bcl-x protein. The intensity of staining and the percentage of immunopositive cells seemed to be directly correlated with the Gleason score, with Gleason score tumors 8-10 and metastatic lesions staining strongest. Furthermore, it is highly likely that the majority of the staining originated with bcl-xL, rather than bcl-x (33), which seems to be infrequently expressed in vivo.

bcl-xL has been shown to be a potent protector of cellular apoptosis induced by metabolic inhibitors and antineoplastic agents (34). When transferred into the murine IL-3-dependent prolymphocytic cell line FL5.12, bcl-xL could greatly ameliorate the proapoptotic effects of bleomycin, cisplatin, etoposide, vincristine, and doxorubicin. Furthermore, bcl-xL was capable of allowing cells to remain cell cycle-arrested until after drug clearance, at which point cellular proliferation could be re-

**DISCUSSION**

bcl-x is a relatively new member of the bcl-2 family of apoptosis-regulating genes. Its expression has been detected in a range of normal tissues, particularly in the central nervous system and thymus. Recent immunocytochemical studies (29) have demonstrated that bcl-xL can be detected in numerous tumor cell lines as well, including human erythroleukemia and K562 cells (30), murine myeloma cells (31), and HCW-2 cells, which are an apoptosis-resistant variant of human promyelocytic leukemia HL-60 cells (32), and in other tumor cell lines and tissues.

**Fig. 8** Immunoblot analysis of bak and bcl-xL expression in prostate cancer cells after treatment with 10 μM estramustine. A, increase in bak expression in LNCaP cells after treatment with 10 μM estramustine for the stated times. Protein (25 μg/lane) was isolated as described in the text, blotted, and probed with an anti-bak polyclonal antibody. Bands were visualized by ECL. B, bcl-xL protein levels remained unchanged in LNCaP cells treated with estramustine. Protein (25 μg/lane) was isolated, blotted, and visualized as described in A. A polyclonal anti-bcl-x antibody was used. C, bak protein levels remained unchanged in PC-3 cells after treatment with estramustine. Protein (25 μg/lane) was isolated, blotted, and visualized as described in A. A polyclonal anti-bak antibody was used.
that is independent of changes in bak expression. However, in PC-3 androgen-independent cells, es-
causes up-regulation of bak expression but no change in bcl-xL expression but no change in
(LNCaP cells only).

controlled by the intracellular content of bcl-xL and bak protein
including bcl-xL and bax. Thus, our data suggest the following
process may be context dependent. In this report, we demon-
strated by gene transfer experiments, bak is a
bcl-2-related gene that functions as a dominant regulator of apoptotic

Finally, we were unable to detect any but minimal levels of
cell line caused specific up-regulation of bak protein expression
and induced the cells to undergo apoptosis without changes in
the expression level of the other apoptosis-regulating genes,
including bcl-xL and bak. Thus, our data suggest the following
scenario:

(a) The rate of apoptosis in LNCaP and PC-3 cells in response to Taxol and/or estramustine may be at least partially controlled by the intracellular content of bcl-xL and bak protein (LNCaP cells only).
(b) Treatment of the LNCaP and PC-3 cells with Taxol causes down-regulation of bcl-xL expression but no change in bak expression. Treatment of LNCaP cells with estramustine causes up-regulation of bak expression but no change in bcl-xL expression. However, in PC-3 androgen-independent cells, estramustine-induced cellular apoptosis can occur by a mechanism that is independent of changes in bak expression.
(c) Our results, in combination with those of Minn et al. (34), suggest that for metastatic prostate cancer, especially for
tumors with a high Gleason score, the sensitivity to Taxol will decrease as bcl-xL levels increase. The combination of estramustine and Taxol has already been described by Speicher et al. (14), as having augmented antimicrotubule activity in prostate cancer cell lines and has significantly increased cellular cytotoxicity versus the single agents. These data, in combination with ours reported above, strongly suggest that the combination of Taxol or a taxane plus estramustine deserves intensive clinical study. Indeed, during the course of this investigation, several patients have already received the combination of taxotere and estramustine as part of a clinical therapeutic trial, with extremely encouraging results.

ACKNOWLEDGMENTS
We thank the reviewers of this paper for helpful comments.

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