Interleukin 6, a Nuclear Factor-κB Target, Predicts Resistance to Docetaxel in Hormone-Independent Prostate Cancer and Nuclear Factor-κB Inhibition by PS-1145 Enhances Docetaxel Antitumor Activity

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Abstract

Purpose: To investigate whether nuclear factor κB (NF-κB)/interleukin 6 (IL-6) was linked to docetaxel response in human prostate cancer cell lines, and whether inhibition of NF-κB sensitized tumor cells to docetaxel. We also aimed to correlate IL-6 (as a surrogate marker of NF-κB) and docetaxel response in hormone-independent prostate cancer (HIPC) patients.

Experimental Design: Hormone-dependent (LNCaP) and hormone-independent (PC-3 and DU-145) prostate cancer cell lines were exposed to docetaxel alone or combined with the NF-κB inhibitor PS-1145 (an inhibitor of IκB kinase-2). Effects of dose, exposure time, and schedule dependence were assessed. Activation of NF-κB was assayed by electrophoresis mobility shift assay and luciferase reporter assay, IL-6 levels by ELISA, and cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell cycle and apoptosis were assessed by fluorescence-activated cell sorting analysis. Apoptosis was also measured by detection of cleavage of poly(ADP-ribose) polymerase. In patients with metastatic HIPC receiving docetaxel-based chemotherapy, IL-6 serum levels were assayed before chemotherapy and every 3 to 4 weeks thereafter.

Results: PC-3 and DU-145 cells had higher NF-κB activity, secreted more IL-6, and were more resistant to docetaxel than LNCaP cells. NF-κB activity was induced by docetaxel. Cotreatment with docetaxel and PS-1145 prevented docetaxel-induced NF-κB activation, reduced IL-6 production, and increased docetaxel effects on cell viability in PC-3 and DU-145 cells but not in LNCaP. Synergism with docetaxel and PS-1145, as assayed by median-effect principle, was observed in DU-145 and PC-3. In HIPC patients, pretreatment IL-6 serum levels correlated to prostate-specific antigen (PSA) response: median IL-6 level was 10.8 ± 9.5 pg/mL in PSA responders versus 36.7 ± 20.8 pg/mL ($P = 0.006$) in nonresponders. A PSA response was also linked to a decline in IL-6 levels during treatment. Median overall survival was 6.8 months in patients with high IL-6 versus 16.6 months in those with low IL-6 ($P = 0.0007$). On multivariate analysis, pretreatment IL-6 ($P = 0.05$) was an independent prognostic factor for time to disease progression and survival.

Conclusions: Inhibition of NF-κB emerges as an attractive strategy to enhance docetaxel response in prostate cancer. The interest of this view is further supported by a significant association between high IL-6 in sera of HIPC patients and decreased response to docetaxel.

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Received 12/16/05; revised 6/12/06; accepted 7/13/06.

Grant support: Spanish Ministry of Science and Technology (MCYT) grants SAF 2003-08181 (A. Rovira, P. Gascon, J. Albanell, and B. Mellado) and GEN2003-20243-C08-08 (J. Albanell); Asociación Española Contra el Cáncer/Catalunya Contra el Cáncer 2002 (A. Rovira, P. Gascon, and J. Albanell); "Premi Fi de Residencia 2003-2004" research grant from the Hospital Clínic of Barcelona, Barcelona, Spain (J.M. Domingo-Domenech); fellowship from la Fundación Científica de la Asociación Española Contra el Cáncer (A. Rovira); and a generous donation from Fundació Cellex (Barcelona) to the Laboratory of Experimental Oncology.

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doi:10.1158/1078-0432.CCR-05-2767

Prostate cancer is the second leading cause of death in men from cancer. Despite the availability of local treatment with radical prostatectomy or radiotherapy, many patients develop disease relapse after primary therapy. The first sign of prostate cancer recurrence is often heralded by an increase of serum prostate-specific antigen (PSA) levels and is known as biochemical relapse. Subsequently, patients can develop clinical relapse of the disease typically featuring bone metastases. Initially, the majority of metastatic prostate cancer patients respond to antiandrogen therapy. However, eventually prostate cancer progresses despite androgen withdrawal, transforming into a hormone-refractory status. Docetaxel-based chemotherapy has recently been shown to improve survival of patients with metastatic HIPC and became a standard treatment in this clinical situation (1, 2). For this reason, knowledge of mechanisms of response or resistance to docetaxel in HIPC would be of interest to select those patients more likely to derive a clinical benefit and to rationally design combination strategies that might improve therapeutic results. Nuclear factor κB (NF-κB) is an attractive candidate to mediate chemoresistance in prostate cancer.

NF-κB/Rel transcription factors are a group of structurally related proteins, with five members in mammals, including p65 (also known as RelA). NF-κB dimers are rendered inactive in nonstimulated cells in the cytoplasm due to its association to NF-κB inhibitory proteins, known as IκBs. The IκB kinase (IKK) complex is the key enzyme in activation of the NF-κB pathway. IKK contains at least three subunits. Two of these subunits, IKK-1 and IKK-2, are serine-threonine kinases. The third subunit, NEMO, is a regulatory factor. A variety of extracellular signals activate NF-κB by generating cellular signals leading to the phosphorylation of IκBs by the IKK complex and subsequent degradation by a proteasome-dependent pathway. Proteasome degradation of IκBs allows NF-κB dimers to translocate to the nucleus. In the nucleus, NF-κB initiates the transcription of a wide variety of genes that code for angiogenic factors, cell adhesion molecules, antiapoptotic factors, and cytokines, which are involved in cell survival, invasion, metastasis, and chemoresistance (3).

Previous work from our group and others has shown that activated NF-κB, as assayed by nuclear p65/NF-κB staining, is absent in normal prostate and prostatic intraepithelial neoplasia lesions, whereas it is commonly present in invasive prostate cancer (4–8). Moreover, NF-κB overexpression or activation predicts a high risk of relapse in patients with localized disease, thus suggesting a role in the clinical behavior of prostate cancer (4–8). Notably, one of the target genes of NF-κB encodes interleukin 6 (IL-6), a cytokine that stimulates prostate cancer cell growth in an autocrine and paracrine manner and is involved in the development and progression of prostate cancer (9–14). In addition to its role in oncogenesis, NF-κB has been associated with chemoresistance in various models (14–17). However, its possible role in docetaxel resistance in prostate cancer is yet poorly characterized.

We hypothesized that NF-κB might counteract, at least in part, the antitumor activity of docetaxel in HIPC and that NF-κB inhibition might sensitize cells to the effects of docetaxel. Here we report that docetaxel induced NF-κB activation and IL-6 production in HIPC cell lines. Furthermore, inhibition of NF-κB by PS-1145 (an IKK-2 inhibitor) prevented docetaxel-induced NF-κB activation and IL-6 production and also enhanced docetaxel antitumor effects. Complementing the preclinical data, we observed that high IL-6 levels in serum of HIPC patients predicted resistance to docetaxel-based chemotherapy and was associated with a shorter survival.

Materials and Methods

Cell cultures. Three human prostate cancer cell lines, PC-3, DU-145, and LNCaP, were obtained from the American Type Culture Collection (Rockville, MD). PC-3 cells were grown in F-12K Nutrient Mixture medium (Life Technologies, Inc., Rockville, MD) and DU-145 and LNCaP were grown in RPMI 1640 (Life Technologies) supplemented with 10% FCS and cultured at 37°C in a humidified air containing 5% CO_2.

Reagents. The IKK-2 inhibitor PS-1145 was provided by Millennium Pharmaceuticals, Inc. (Cambridge, MA) and docetaxel was purchased from Sigma (St. Louis, MO). Both agents were dissolved at 10 nmol/L in DMSO before dilution in cell culture medium.

IL-6 assay. IL-6 was assayed in conditioned medium and serum samples and quantified with a commercially available colorimetric ELISA. IL-6 blood samples were collected in sterile tubes not containing anticoagulants. Samples were centrifuged at 3,000 rpm for 10 minutes and the plasma stored at −70° until processing. The assay (Biosource Europe, Nivelles, Belgium) is based on an oligoclonal system in which a mix of monoclonal antibodies directed against distinct epitopes of IL-6 is used. Standards used in the calibration curve and samples containing the cytokine react with capture monoclonal antibodies coated on the microtiter well. After incubation, excess of antigen was removed by washing. A second monoclonal antibody labeled with horseradish peroxidase was added. After incubation, the microtiter plate was washed and bound enzyme-labeled antibodies were measured through a chromogenic reaction. The reaction is stopped with the addition of H_2SO_4 as stop solution. The colorimetric determination was done by means of a polychromic reader (Vmax EASIA reader, Medgenix Diagnostics, Fleurus, Belgium). The sensitivity of this assay allows the detection of IL-6 levels as low as 1 pg/mL and an assay range of 10 to 400 pg/mL. Standards of known IL-6 content and control serum specimens were analyzed jointly with unknown samples. Absorbance was read with a microplater reader at 450 nm and was directly proportional to the concentration of IL-6 present in the samples. Concentrations of cytokines from samples were determined comparing the absorbances of the samples to the standard curves. All samples were assayed in duplicate and the procedure was done according to the instructions of the manufacturer.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. LNCaP, DU-145, and PC-3 cells were seeded in 96-well plates the day before treatment at a density of 1×10^4, 5×10^3, and 1×10^4 cells per well, respectively. Various combinations of docetaxel and PS-1145 were added to cells and, 72 hours later, cell viability was assessed using the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS) Kit (Promega, Madison WI) according to the protocol provided, and absorbance was measured on a microplate spectrophotometer (Molecular Dynamics, Sunnyvale, CA) at 490 nm (test wavelength) and 690 nm (reference wavelength). The percentage of surviving cells was estimated by dividing the $A_{490\ nm} - A_{690\ nm}$ of treated cells by the $A_{490\ nm} - A_{690\ nm}$ of control cells. IC_{50} values were estimated from the dose-response curve. Data were derived from at least three independent experiments (in quatriplicate). Combination assays were done as a simultaneous schedule (72 hours of concomitant exposure to both agents) or sequential schedules (docetaxel was given first for 24 hours, PS-1145 was then administrated, and cells were incubated for 48 hours or vice versa). Culture supernatants were collected, cleared by centrifugation, and stored at −70°C until assayed for IL-6 levels.

To assess the nature of the interaction observed between docetaxel and PS-1145, additional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done and analyzed with the

software Calcusyn, which uses the median effect method of Chou and Talalay (18). Data analysis through this method generates a coefficient of interaction for each drug concentration combination, with a coefficient of interaction < 1 indicating a synergistic interaction between two drugs (i.e., greater then the expected additive effect when two agents are combined), additive when coefficient of interaction = 1, and antagonistic when coefficient of interaction > 1 (i.e., less than the expected additive effect when two agents are combined). For *in vitro* combination assays, cells were incubated with drugs both individually and together at multiple drug concentration combinations. Because PS-1145 alone slightly affected cell viability (IC_{50} no reached), a docetaxel/PS-1145 concentration ratio was arbitrarily fixed at 1:1,000 for these experiments.

Trypan blue exclusion assay. For the trypan blue exclusion test, cells were treated and cultured as described. Cells were harvested and trypan blue dye solution was added to the cell suspensions. Total and viable cell counts (survival rate) were determined with standard hemocytometer procedure. Live viable cells were seen as colorless (impermeable to the dye due to intact membrane) and dead cells were seen as blue (permeable to dye due to disruption of cell membrane).

Apoptosis. Analysis of apoptosis by flow cytometry. Apoptosis was measured by surface Annexin V staining. To analyze apoptosis, cells were harvested by trypsinization, washed in PBS, and incubated in 100 μL of Annexin-binding buffer with 1 μL of Annexin V-FITC (Bender MedSystems, Vienna, Austria) for 15 minutes in the dark. An equal volume of Annexin-binding buffer with 1 μL of 7-amino-actinomycin D (Sigma-Aldrich, Steinheim, Germany) was added just before analysis. Cells were acquired and analyzed in a FACSCalibur cytometer with Cell Quest software (Becton Dickinson, Mountain View, CA).

Poly(ADP-ribose) polymerase cleavage detection method. To study poly(ADP-ribose) polymerase proteolysis, adherent and detached cells were combined for whole-cell protein extract preparations. Poly(ADP-ribose) polymerase cleavage was estimated by immunoblotting analysis with anti–poly(ADP-ribose) polymerase antibody (PharMingen, San Diego, CA).

Cell cycle analysis. Cell cycle was measured as DNA content by flow cytometry. Cells were harvested by trypsinization, washed in PBS, and fixed in cold 70% ethanol at 4°C overnight. Cells were then washed twice in PBS and resuspended in PBS at a final concentration of 50 mg/mL propidium iodide and 100 μg/mL RNase (both from Sigma-Aldrich). At the indicated time points, samples were analyzed by a FACSCalibur flow cytometer using Cell Quest software (Becton Dickinson).

Electrophoresis mobility shift assay. Nuclear extracts were prepared as described elsewhere (15). The binding of NF-κB was determined by electrophoresis mobility shift assay using a specific NF-κB consensus oligonucleotide probe (5-AGTTGAGGGGACTTTCCCAGGC-3) labeled with 25 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham Biosciences, Buckinghamshire, United Kingdom) and T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN). The labeled probes were purified in a G-25 spin column (Amersham Biosciences). Nuclear extracts (5 μg of protein) were incubated with the ^{32}P-labeled oligonucleotide probe (10 ng) for 30 minutes at room temperature in 20 μL of binding buffer [20 mmol/L HEPES (pH 7.9), 59 mmol/L KCl, 1 mmol/L DTT, 0.5 mmol/L EDTA, 10% glycerol, 1 mg/mL bovine serum albumin, 0.2% NP40, 50 ng of poly(dI-dC)]. Reaction was stopped by adding 1 μL of gel loading buffer. Samples were run in 0.5× Tris-borate EDTA buffer on nondenaturing 4% polyacrylamide gels to separate the DNA-protein complexes from unbound oligonucleotides. The specificity of NF-κB band was confirmed by competition experiments with the addition of unlabeled, either specific or unrelated (acid phosphatase 2), oligonucleotides (100-fold excess) in the DNA-protein binding reaction. Gels were vacuum-dried and exposed to X-ray film (Kodak X-OMAT) with 2×10^5 cells per well with an intensifying screen at −80°C.

Transfections and NF-κB luciferase reporter assay. PC-3 cells (2×10^5 per well) were plated in 35-mm dishes and cotransfected the next day using Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA) according to the protocol of the manufacturer done with 1 μg of total DNA, consisting of 900 ng of a NF-κB-luciferase reporter plasmid (kindly provided by Dr. Joan Gil, Barcelona University, Barcelona, Spain) and 100 ng of empty vector pCDNA3.1-neo^r (Invitrogen). Cells were diluted and transferred to 100-mm dishes and stable transfectants were then selected in F-12K Nutrient Mixture medium (Life Technologies) supplemented with 10% fetal bovine serum and 500 μg/mL G418 (Sigma). A pool of selected cells was collected and used for experiments. Stable PC-3 cells expressing NF-κB-luc were seeded at 2×10^5 per well in a 35-mm well plate. Twenty-four hours later, cells were treated with different combinations of reagents. The preparation of cell lysates and luciferase activity measurements were made with the Luciferase Assay Kit (Promega) according to the instructions of the manufacturer. Relative light units were measured in a TD-20/20 luminometer (Clontech laboratories, Inc., Palo Alto, CA). The luciferase activities were normalized to protein contents. Data shown are from one of at least two independent experiments with similar results.

Patients. Patients with metastatic HIPC receiving standard first-line docetaxel-based chemotherapy were included in the study and were prospectively tested for IL-6 in serum before and during chemotherapy treatment. The primary objective of the study was to test if IL-6 in serum, as a surrogate marker of NF-κB activation, predicted resistance to docetaxel in patients with metastatic HIPC. IL-6 results were correlated with clinical characteristics and outcome of patients. Inclusion criteria were histologically proven adenocarcinoma of the prostate and evidence of disease progression (PSA increase on two consecutive measurements 2 weeks apart, in addition to either new lesions on bone scan or increase in the size of measurable lesions) in the presence of castrate levels of testosterone (<50 ng/mL). Patients who received antiandrogen therapy with flutamide or bicalutamide had to show increasing PSA levels 6 weeks after antiandrogen withdrawal. Patients who had not undergone orchiectomy were required to continue gonadal ablation with a luteinizing hormone–releasing hormone analogue. Patients had to have an Eastern Cooperative Oncology Group performance status of 0 to 2, appropriate hepatic and renal function at baseline, and they were required to give written informed consent for study entry. Patients with infectious or inflammatory diseases were not eligible.

Pretreatment evaluation included a complete medical history, physical examination, bone scan, computed tomography of the abdomen and pelvis, and laboratory studies, including PSA, testosterone, cell blood count, and biochemistry. Follow-up evaluation included physical examination and laboratory studies [complete blood count, PSA, acid phosphatase, lactate dehydrogenase (LDH), and IL-6 serum levels] before each cycle of chemotherapy and every 3 to 4 weeks after treatment was stopped. Bone scan and computed tomography (if measurable disease) were done every 3 months during therapy or when clinically indicated. Treatment consisted of docetaxel at a dose of 70 mg/m^2 i.v. day 2, plus estramustine 280 mg/d p.o., days 1 to 5, every 21 days; or docetaxel 36 mg/m^2 i.v. 3 consecutive weeks, every 28 days. Patients received a maximum of six cycles of chemotherapy unless disease progression or unacceptable toxicity was observed. All patients were evaluated for PSA response, time to PSA disease progression, and survival. The criteria for PSA response were based on the guidelines from the PSA Working Group (19). PSA response was considered as a decline of PSA >50% from the baseline. PSA-progression as an increase of PSA >25% or an increase in the absolute-value PSA level by at least 5 ng/mL from the baseline or from the nadir (in case of previous decrease) confirmed in a second determination. Time to PSA progression was considered from the time treatment started until PSA progression was detected. Overall survival was defined as the time from start of treatment to patient death. For IL-6 determination, aliquots of serum samples were obtained before the start of docetaxel and every 3 to 4 weeks during therapy. These samples were kept at −70°C until assayed. This study was reviewed and approved by the Ethics Committee of our institution.

Statistical analysis. Statistical analysis was carried out with SPSS version 11.0 (SPSS, Inc., Chicago, IL). Correlation tests between continuous quantitative variables (Spearman correlation test), continuous quantitative and qualitative variables (*t* test), and qualitative variables (χ^2 test) were used to determine the significance of *in vitro* experiments and the association between IL-6 baseline serum levels and prognostic variables. Time to biochemical disease progression and overall survival were analyzed by the Kaplan-Meier method. Curves were compared by the log-rank test. Univariate and multivariate analysis including continuous and qualitative clinical-pathologic variables was done with the Cox proportional hazards model. All the statistical tests were conducted at the two-sided 0.05 level of significance.

Results

NF-κB constitutive activity, IL-6 levels, and docetaxel sensitivity in prostate cancer cells. NF-κB DNA-binding activity of hormone-dependent (LNCaP) and hormone-independent (PC-3 and DU-145) human prostate cancer cell lines was assayed by electrophoresis mobility shift assay. Constitutive NF-κB activity was detected in androgen-independent PC-3 and DU-145 cells, whereas no constitutive NF-κB activation was detected in the hormone-dependent cell line LNCaP (Fig. 1A). IL-6 production in conditioned medium was detected in DU-145 and PC-3 cell lines but was undetected in the medium of LNCaP cells (data not shown).

The hormone-dependent cell line LNCaP, without constitutive NF-κB activity and undetected IL-6 production, showed the highest sensitivity to docetaxel, as determined by MTT assay (IC_{50} range, 2.5-5 nmol/L). On the other hand, the hormone-independent cell line PC-3 with the highest constitutive NF-κB activity and baseline IL-6 secretion was the most resistant to docetaxel (IC_{50} range, 50-60 nmol/L) whereas DU-145 showed an intermediate sensitivity to docetaxel (IC_{50} 5-20 nmol/L; Fig. 1B).

PS-1145 sensitized hormone-refractory prostate cancer cells to docetaxel. Time-response experiments with docetaxel in PC-3 cells, at a concentration of 60 nmol/L (that corresponds to the estimated mean IC_{50} in PC-3 cells), showed NF-κB DNA-binding activity at 6 hours of drug exposure (data not shown). We then tested the ability of PS-1145 to prevent NF-κB activation. Both baseline and docetaxel-induced DNA binding of NF-κB were strongly reduced by PS-1145 in PC-3 and DU-145 cells (Fig. 1C). Drug effects on NF-κB transcriptional activity were then assayed in a pool of stably transfected PC-3 cells expressing NF-κB luciferase. The IKK-2 inhibitor PS-1145 significantly inhibited constitutive activation of NF-κB and decreased NF-κB activation induced by docetaxel (Fig. 1D).

Based on the observations mentioned earlier, we tested whether inhibition of NF-κB by PS-1145 sensitized prostate cancer cells to docetaxel. LNCaP, DU-145, and PC-3 were treated with PS-1145 (20 μmol/L) alone or in combination with docetaxel over 72 hours. PS-1145 as a single agent reduced cell viability by <20% as assayed by MTT. In the hormone-dependent cell line LNCaP, the combination of PS-1145 and docetaxel was not superior to docetaxel alone (Fig. 2A). However, when PS-1145 was combined with docetaxel in hormone-independent prostate cancer cells, a significant decrease in cell viability was observed compared with docetaxel alone (Fig. 2B and C). Sequential treatments with docetaxel for 24 hours followed by PS-1145 for 48 hours, or PS-1145 for 24 hours followed by docetaxel for 48 hours, had inferior or similar effects than simultaneous treatment (docetaxel plus PS-1145 for 72 hours), as assayed by MTT (data not shown). The nature of the interaction between PS-1145 and docetaxel in

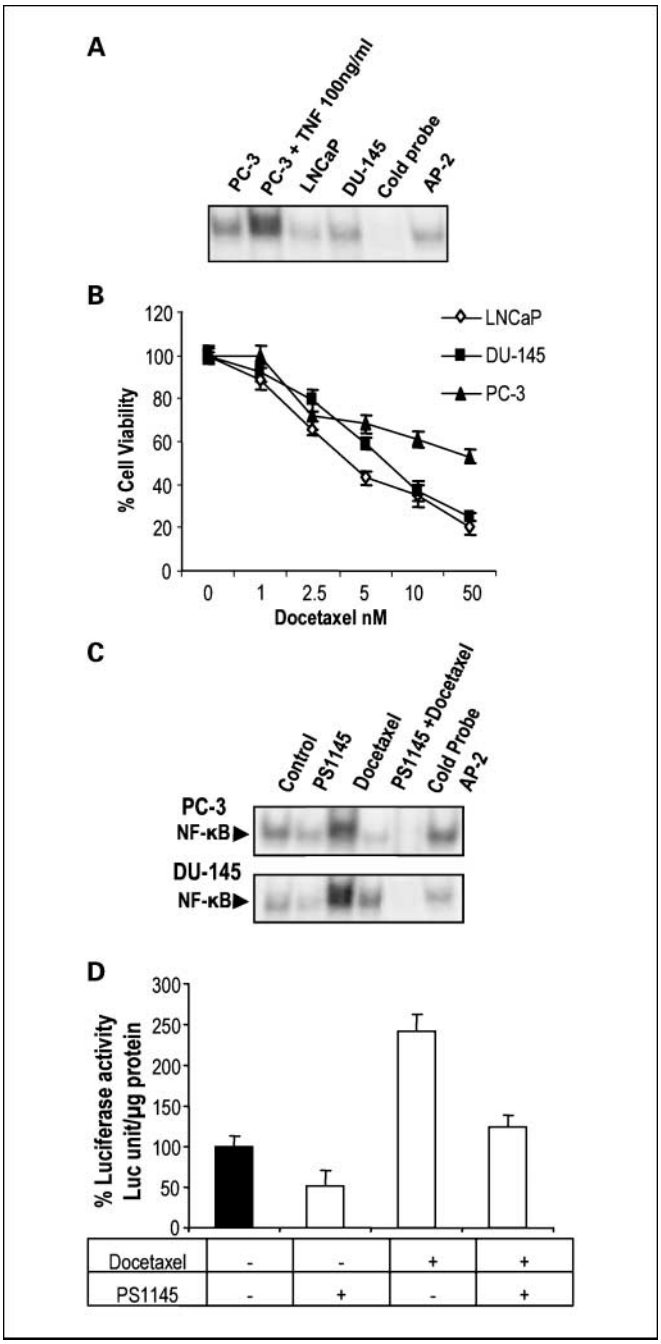

Fig. 1. *A,* electrophoresis mobility shift assay of prostate cancer cell lines. TNF-α addition in PC-3 cells was used as a positive control and a cold probe and acid phosphatase-2 as negative controls. *B,* MTT assay of docetaxel in prostate cancer cell lines at 72 hours of exposure. *C,* PS-1145 inhibited basal and docetaxel-induced NF-κB DNA-binding activity and NF-κB transcriptional activity. PC-3 cells were treated with 20 μmol/L PS-1145 alone for 24 hours, 60 nmol/L docetaxel for 6 hours, and 20 μmol/L PS-1145 for 18 hours plus 60 nmol/L docetaxel for 6 hours. DU-145 cells were treated with 20 μmol/L PS-1145 alone for 24 hours, 20 nmol/L docetaxel for 8 hours, and 20 μmol/L PS-1145 for 16 hours plus 60 nmol/L docetaxel for 8 hours. *D,* effect of PS-1145 and docetaxel on NF-κB transcriptional activity in PC-3 stably transfected luciferase reporter cells.

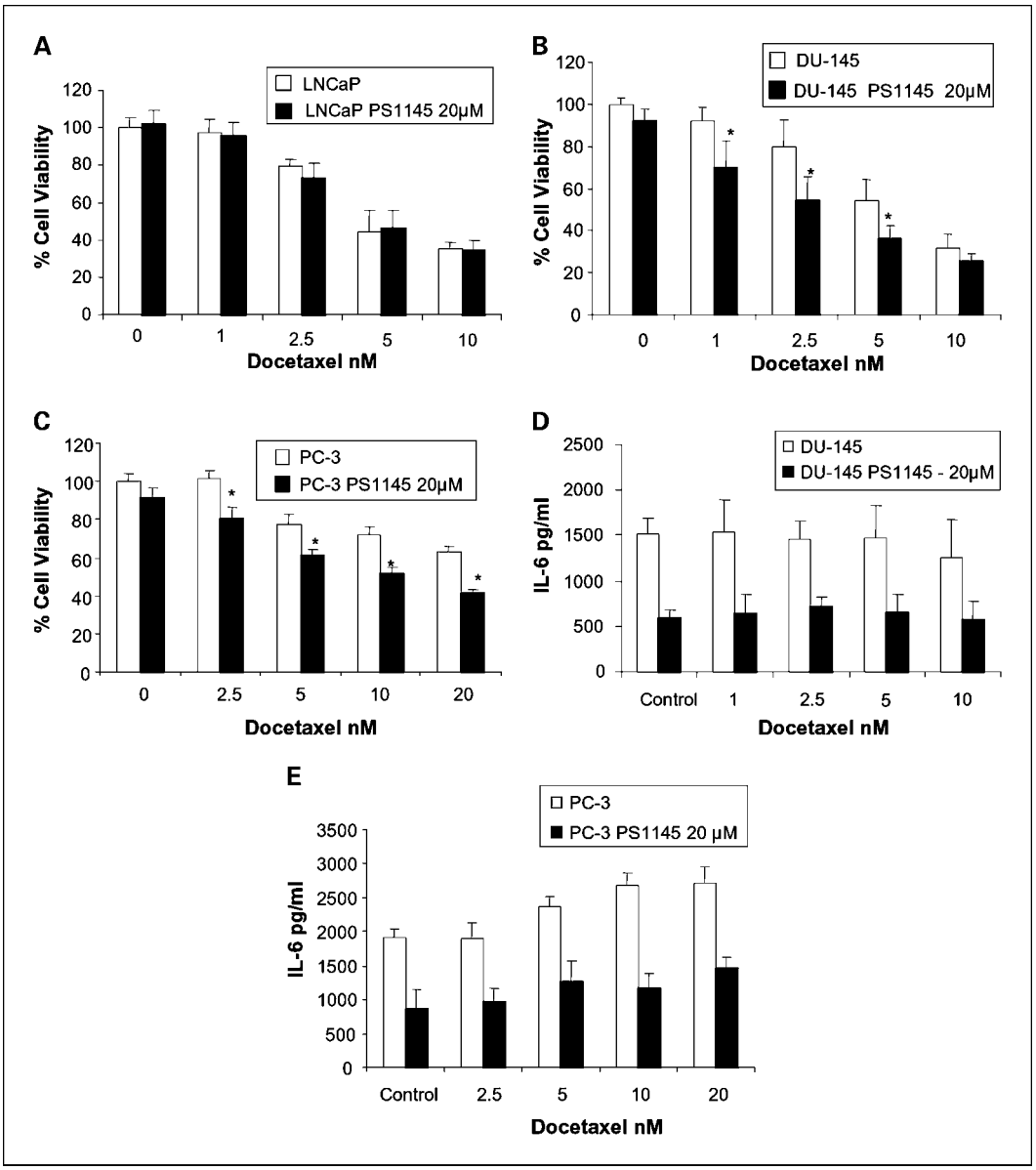

Fig. 2. Prostate cancer cell lines (LNCaP, DU-145, and PC-3) were treated with 20 μmol/L PS-1145 and increasing concentrations of docetaxel for 72 hours. Cell viability was assessed by MTT assay and IL-6 protein expression in culture medium was measured by ELISA. *A,* no significant decrease in cell viability was observed in LNCaP cancer cells when exposed to combined treatment. *B* and *C,* cell viability of DU-145 and PC-3 cancer cells significantly decreased when PS-1145 was combined with docetaxel. *D* and *E,* IL-6 medium levels of DU-145 and PC-3 cancer cells exposed to PS-1145 significantly decreased compared with control or docetaxel alone. Independent experiments were made in triplicates. *, $P \leq 0.05$ (*t* test).

PC-3 and DU-145 cells was analyzed by median effect and isobologram analyses. Simultaneous exposure to PS-1145 and docetaxel induced a synergistic interaction in PC-3 cells. In DU-145 cells, the combination resulted in either additive or synergistic effects (Table 1).

IL-6 levels, as a surrogate marker of NF-κB activity, were also assayed in conditioned media of prostate cancer cell lines exposed to PS-1145 and docetaxel, alone or in combination. PS-1145 produced a dose-dependent reduction of IL-6 production in DU-145 and PC-3 at 72 hours of exposure (data not shown). In cells exposed to docetaxel, IL-6 levels in conditioned media remained high and essentially unchanged in DU-145 cells (Fig. 2D). In PC-3 cells, docetaxel induced a dose-dependent increase of IL-6 production (Fig. 2E). Combination therapy with docetaxel and PS-1145 reduced IL-6 levels in DU-145 cells and prevented docetaxel-induced increase of IL-6 in PC-3 cells (Fig. 2D and E). In LNCaP cells, with no baseline IL-6 production, IL-6 remained undetected in media after docetaxel and/or PS-1145 exposure (data not shown).

PS-1145 alone had no effect on cell cycle in both DU-145 and PC-3 cells analyzed at 24 and 48 hours. In DU-145 cells, cell cycle analysis showed a G_2-M arrest induced by docetaxel (5 nmol/L) at 24 and 48 hours, and this effect was not modified by the addition of PS-1145 (20 μmol/L). In PC-3, a G_2-M accumulation was observed at 24 hours with docetaxel (20 nmol/L), with no additional effect induced by PS-1145 (20 μmol/L). At 48 hours, a shift to G_0-G_1 phase accumulation was observed and this effect was not modified by the combination (data not shown). By flow cytometry analysis, PS-1145 (20 μmol/L) alone did not induce apoptosis in both PC-3 and DU-145 cells (Fig. 3A and B). Docetaxel (5 nmol/L) induced apoptosis in 20% (±8% SD) and 38.05% (±8.3% SD) of DU-145 cells at 48 and 72 hours, respectively. This effect was slightly higher with the combination (26.7 ± 9.9% and 40.78 ± 11.3% at 24 and 48 hours) but it did not reach

Table 1. Median effect analysis of the interaction of docetaxel and PS-1145 in PC-3 and DU-145 cells

	Docetaxel (nmol/L)	PS-1145 (μmol/L)	Combination index	Description
PC-3	1	1	0.524	Synergism
	2.5	2.5	0.352	Synergism
	5	5	0.538	Synergism
	10	10	0.679	Synergism
	20	20	0.890	Slight synergism
DU-145	1	1	0.689	Synergism
	2.5	2.5	0.798	Moderate synergism
	5	5	0.840	Moderate synergism
	10	10	0.837	Moderate synergism
	20	20	1.023	Nearly additive

statistical significance (Fig. 3B). In PC-3, docetaxel (20 μmol/L) induced apoptosis in 6.28% (±2.6% SD) and in 12.4% (±1.3% SD) of the cells at 48 and 72% hours, respectively, and this effect was not increased by the addition of PS-1145 (Fig. 3A). Poly(ADP-ribose) polymerase cleavage did not show a marked induction of apoptosis by PS-1145 alone or an increase in docetaxel-induced apoptosis when tested at 24 hours, 48 hours (Fig. 3C), and 72 hours (data not shown) of drug exposure. After 72 hours of docetaxel exposure, alone or with PS-1145, PC-3 cells exhibited an enlarged volume and the presence of cytoplasmic vacuoles. Trypan blue test revealed an increased percentage of cell death in cells treated with docetaxel plus PS-1145 compared with docetaxel alone in both PC-3 and DU-145 cells (data not shown).

High IL-6 serum levels predict resistance to docetaxel in patients with HIPC. Thirty-seven patients were included in the study (Table 2). At the time of this analysis, all of them had progressed to docetaxel chemotherapy and 26 (70.3%) patients had died due to prostate cancer progression. Median follow-up time was 21.9 months (range, 3.5-38.8 months). PSA response was achieved in 19 (51.4%) patients, disease stabilization in 9 (24.3%), and PSA progression in 9 (24.3%). Median time to PSA progression was 4.2 months (range, 0.6-15.1 months) and median survival was 9.7 months (range, 2.0-38.4 months).

Median baseline IL-6 level was 16.0 pg/mL (range, 0-156 pg/mL) and this value was chosen as a cutoff for further analysis. Low IL-6 was defined as serum levels <16 pg/mL (n = 22 patients) and high IL-6 as >16 pg/mL (n = 15). By univariate analysis, baseline IL-6 correlated with LDH (r = 0.63, P < 0.0001, Spearman correlation test), number of bone metastasis (r = 0.33, P = 0.045, Spearman correlation test), and inversely with hemoglobin levels (r = −0.36, P = 0.029, Spearman correlation test) and PSA response (P = 0.006). In patients with PSA-response, mean baseline IL-6 level was 10.8 ± 9.5 pg/mL, whereas in patients who did not respond, mean IL-6 level was 36.7 ± 20.8 pg/mL. Moreover, we analyzed IL-6 levels as a binary variable (IL-6 <16 pg/mL or IL-6 >16 pg/mL) and we observed that baseline IL-6 correlated with PSA-response. Among the 22 patients with baseline IL-6 <16 pg/mL, 15 (68.2%) responded whereas only 4 of 15 (26.6%) patients with IL-6 >16 responded to docetaxel (P = 0.013, χ^2 test).

By univariate analysis, the number of bone metastasis (P = 0.003), localization of metastasis (visceral versus nonvisceral, P = 0.002), pretreatment LDH (P < 0.0001), hemoglobin (P = 0.003), and baseline IL-6 (P < 0.0001) were predictive factors for time to PSA progression. Moreover, IL-6 levels analyzed as a binary variable were also associated with time to PSA progression (Fig. 4A). Median time to PSA progression was 5.6 months for patients with IL-6 ≤16 pg/mL versus 2 months for those with IL-6 >16 pg/mL (P < 0.0001, log-rank test). In the multivariate analysis, pretreatment IL-6 level was the only independent prognostic factor for time to PSA progression. Furthermore, when IL-6, hemoglobin, and LDH levels were included in the multivariate analysis as quantitative variables, IL-6 levels continued to be the sole independent prognostic factor (P = 0.006). For overall survival, in the univariate analysis, localization of metastasis (P = 0.004), LDH levels (P < 0.0001), hemoglobin levels (P = 0.007), and IL-6 levels (P < 0.0001) were significant prognostic factors. Binarized IL-6 levels differentiated two groups of patients (Fig. 4B): patients with baseline IL-6 ≤16 pg/mL had a median overall survival of 16.6 months whereas patients with IL-6 >16 pg/mL had a median overall survival of 6.8 months (P = 0.0007). In the multivariate analysis, IL-6 level (P = 0.05) was the only independent prognostic factor for survival. When IL-6 was analyzed as a continuous variable in a multivariate analysis, IL-6 (hazard ratio, 1.047; P = 0.004) and the number of bone metastasis (hazard ratio, 1.052; P = 0.049) were independent prognostic factors for PSA progression, and IL-6 (hazard ratio, 1.025; P = 0.047) was the only independent prognostic factor for survival.

Thirty-five assessable patients had serial IL-6 measurements during treatment. Two patients were excluded from the IL-6 serial analysis: one who experienced an acute respiratory infection during therapy that produced a transient increase of IL-6, and a second patient who showed a progression after first chemotherapy cycle and no IL-6 determination was done. Analysis of IL-6 evolution based on previously published criteria showed that a ≥80% decrease in IL-6 during therapy with dexamethasone correlated with biochemical response (20). Among 35 patients, 20 (57.1%) showed a decrease of ≥80% in IL-6, and IL-6 serial changes under therapy correlated with biochemical response. Fifteen of 18 (83.3%) patients with PSA response showed also a decrease in IL-6 level of >80% (P = 0.001, χ^2 test).

Discussion

In the present study, we show that inhibition of NF-κB by the IKK-2 inhibitor PS-1145 abrogates constitutive and docetaxel-induced NF-κB activity and increases docetaxel sensitivity in hormone-independent but not in hormone-dependent prostate

cancer cells. In hormone-independent cell lines, PS-1145 decreases baseline and docetaxel-induced IL-6 production. These preclinical studies indicating a role for NF-κB and IL-6 in docetaxel response are complemented by studies in patients with HIPC. More specifically, in our series of HIPC patients, high IL-6 serum levels predict resistance to docetaxel and a shorter survival.

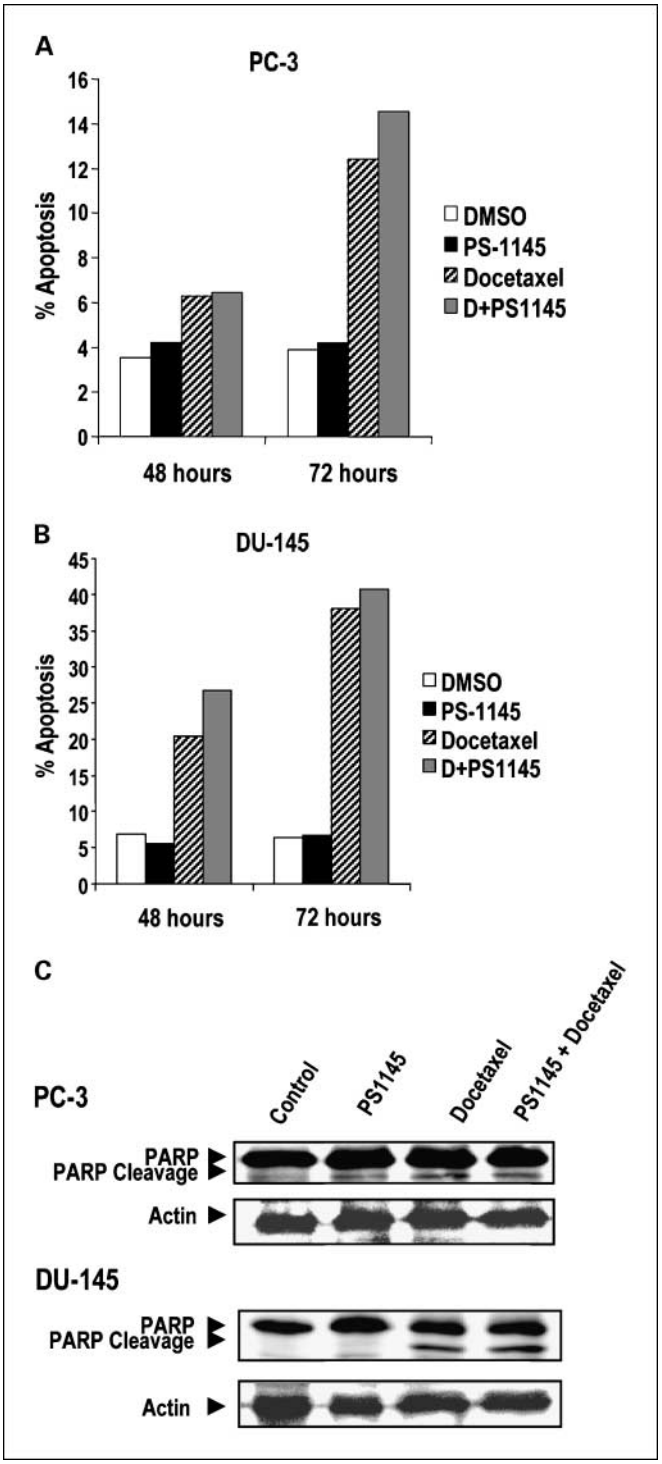

Fig. 3. Analysis of apoptosis by flow cytometry, measured by surface Annexin V staining of PC-3 (*A*) and DU-145 (*B*) cells treated with PS-1145 (20 μmol/L), docetaxel (20 μmol/L in PC-3 and 5 μmol/L in DU-145), or docetaxel plus PS-1145 for 24 and 48 hours. *C*, Western blot of poly(ADP-ribose) polymerase (*PARP*) cleavage of PC-3 and DU-145 cells treated under the same conditions for 48 hours. Independent experiments were made in triplicates.

Table 2. Patients' characteristics

Mean age (y)	67.0 (range, 54-79)
Median time of hormone-dependence (mo)	20.3 (range, 2.3-113.6)
Docetaxel administration	
Every 3 wk	25 patients (67.6%)
Weekly	12 patients (32.4%)
Primary Gleason score	
4	1 patient (2.7%)
5	2 patients (5.4%)
6	4 patients (10.8%)
7	17 patients (45.9%)
8	2 patients (5.4%)
9	10 patients (27.0%)
10	1 patient (2.7%)
Perfomance status	
0	11 patients (29.7%)
1	23 patients (62.2%)
2	3 patients (8.1%)
Disease localization	
Locally advanced	3 patients (8.1%)
Bone ± lymph-nodes	31 patients (83.8%)
Bone + visceral	3 patients (8.1%)
Median no. bone metastasis	11.0 (range 0-31)
IL-6 level	
Median (pg/mL)	16.0 (range, 0-156)
IL-6 ≤ 16 pg/mL	22 patients (59.5%)
IL-6 > 16 pg/mL	15 patients (40.5%)
Median PSA level (ng/mL)	96.0 (range, 5-1,791)
Median acid phosphatase level (IU/L)	417 (range, 93-7,402)
Median LDH level (IU/L)	389 (range, 196-6,353)
Median hemoglobin level (g/L)	13.0 (range, 7-15)

NF-κB controls the expression of numerous gene products that play crucial roles in cell survival, cell cycle, immune responses, angiogenesis, and nervous system function. In most cell types, NF-κB is maintained in a latent form in the cytoplasm through an interaction with the inhibitory IκB proteins. A variety of extracellular signals activate NF-κB by generating cellular signals leading to the phosphorylation of IκBs by the IKK complex and their degradation by the proteasome. Release of IκBs allows the active NF-κB complex to move into the nucleus and activate the responsive genes. Genes that encode antiapoptotic factors are important physiologic targets of NF-κB, playing a crucial role in the regulation of antiapoptotic genes (17, 21). The activation of prosurvival genes will block apoptosis and thus allow tumor cells to evade or limit apoptosis induced by chemotherapy. Several observations support this view. For instance, resistance of human cervical carcinoma cells to cisplatin is partly mediated via enhancement of cisplatin-induced NF-κB activation (15). Inhibition of NF-κB increased the sensitivity of cancer cells to radiation therapy (22) and chemotherapeutic agents (16, 17).

One of the targets of NF-κB is IL-6. The IL-6 promoter consists of at least four transcription factor binding sites, including the IL-6-NF-κB regulatory site. Although the transcriptional regulation of IL-6 expression is quite complex, involving multiple signaling pathways and transcription factors, NF-κB seems to play a key role in autocrine IL-6

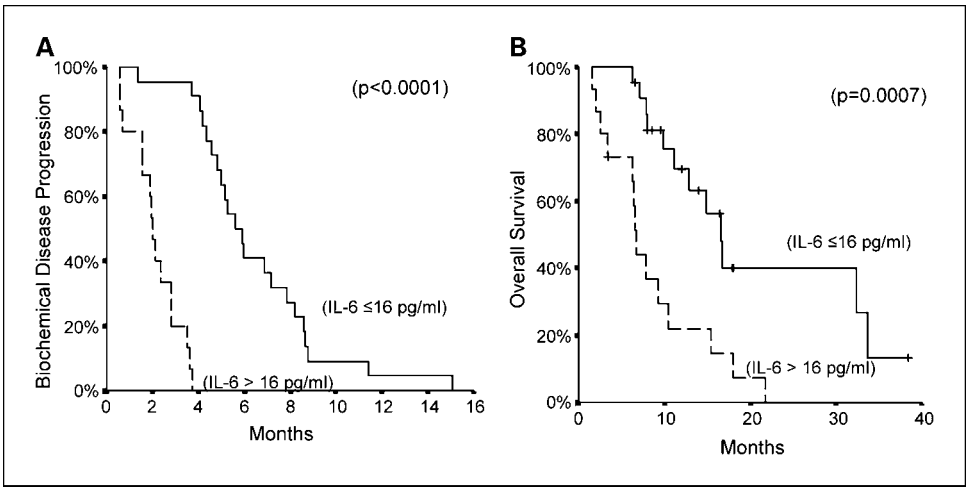

Fig. 4. Biochemical disease progression and overall survival Kaplan-Meier curves for IL-6 serum levels (≤16 and >16 pg/mL) in hormone-refractory prostate cancer patients. *A,* median biochemical disease progression time was 5.6 months for patients with IL-6 ≤16 pg/mL and 2 months for patients with IL-6 >16 pg/mL ($P < 0.0001$). *B,* median overall survival time was 16.6 months for patients with IL-6 ≤16 pg/mL and 6.8 months for patients with IL-6 >16 pg/mL ($P = 0.0007$).

production in prostate cancer cells. In addition, molecular intervention targeting NF-κB is sufficient to block IL-6 expression (10). IL-6 in prostate cancer cell lines PC-3 and DU-145 has been shown to stimulate cell growth in an autocrine and paracrine manner. IL-6 and IL-6 receptor are expressed in prostate cancer and seem to have a role in the acquisition of hormone resistance (13).

In the present study, we report that docetaxel induced NF-κB DNA-binding activity in hormone-independent prostate cancer cells, thus raising the possibility that NF-κB might limit the antitumor activity of docetaxel in prostate cancer. On the other hand, the IKK-2 inhibitor PS-1145 decreased NF-κB DNA-binding activity and IL-6 production in both PC-3 and DU-145 cells. Of note, PS-1145 alone had minimal effects on cell viability, whereas the combination with docetaxel increased antitumor activity with respect to chemotherapy alone in HIPC cell lines. A recent study showed that genistein and p65 small interfering RNA exerted inhibitory effects on p65 expression and NF-κB DNA-binding activity to a similar degree and abrogated the activation of NF-κB stimulated by docetaxel in PC-3 cells (17). It should be noted that the effects of genistein in tumor cells are complex and may affect different cellular pathways, including estrogenic and/or antiestrogenic activities, and inhibition of several protein tyrosine kinases, including epidermal growth factor receptor and Src tyrosine kinase.

In our work, we used a small-molecule IKK-2 inhibitor, PS-1145. Yelelyanov et al. (23) characterized the effects of PS-1145 in hormone-independent prostate cancer cells. In their study, PS-1145 induced caspase-3/caspase-7–dependent apoptosis and significantly sensitized prostate cancer cells to apoptosis induced by tumor necrosis factor α, inhibited cell proliferation, inhibited IL-6, cyclin D1, cyclin D2, inhibitor of apoptosis (IAP)-1, and IAP-2 gene expression, and decreased IL-6 protein level. In addition, PS-1145 inhibited the invasion activity of highly invasive PC-3-S cells. In the work presented here, we showed by luciferase and electrophoretic mobility shift assays that PS-1145 efficiently inhibits both basal and docetaxel-induced NF-κB activity in PC-3 and DU-145 cells. We observed that PS-1145 increased the effects on cell viability induced by docetaxel in these cell lines. The enhancement of apoptosis was modest and did not reach statistical significance. Further experiments showed that PS-1145 reduced IL-6 levels and increased the sensitivity to docetaxel chemotherapy. These results are consistent with our hypothesis supporting a role of NF-κB in limiting the antitumor activity of docetaxel and the potential effectiveness of inhibiting NF-κB as a strategy to sensitize cells to docetaxel. We are currently planning to test the efficacy of this strategy to sensitize prostate cancer cells to docetaxel in *in vivo* models with the ultimate goal of clinical testing.

Measurement of IL-6 in serum of patients has recently emerged as a potential marker of response to various therapies in prostate cancer (20, 24, 25). In one study, done in 25 patients treated with low-dose dexamethasone after progression to androgen ablation, response to dexamethasone was linked to a >80% decrease of serum IL-6 (20). In contrast, none of the nonresponders showed remarkable IL-6 suppression. In a phase I trial of the proteasome inhibitor bortezomib, PSA responses in patients with prostate cancer were observed only in the subgroup of patients with a decline in elevated pretreatment serum IL-6 level, suggesting a NF-κB-mediated effect (24). In a large Cancer and Leukemia Group B 9480 study in metastatic HIPC patients treated with suramin, IL-6 in plasma was collected at baseline (before suramin therapy) from 191 patients and its prognostic significance was analyzed. In this study, plasma IL-6 level was an independent prognostic factor for survival (25).

We here present novel clinical data on IL-6 levels in patients with metastatic HIPC treated with docetaxel-based chemotherapy. In our study, the level of IL-6 before the start of docetaxel chemotherapy correlated with PSA response. Moreover, patients with high IL-6 before the start of chemotherapy had a lower overall survival compared with patients with low IL-6 (median overall survival of 6.8 and 16.6 months, respectively). Basal IL-6 was an independent prognostic factor for survival by multivariate analysis. Moreover, the analysis of IL-6 changes under therapy also suggested a correlation between a PSA response and a decrease of IL-6. These findings support the use of IL-6 levels in prognostic models and add rationale for IL-6-targeted therapy in patients with HIPC.

In summary, our preclinical and clinical results support a role of NF-κB in resistance to docetaxel in HIPC and support the research on NF-κB-targeted therapies to enhance the antitumor activity of docetaxel in this patient population.

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Clinical Cancer Research

Interleukin 6, a Nuclear Factor-κB Target, Predicts Resistance to Docetaxel in Hormone-Independent Prostate Cancer and Nuclear Factor-κB Inhibition by PS-1145 Enhances Docetaxel Antitumor Activity

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Clin Cancer Res 2006;12:5578-5586.