The Novel Antimicrotubule Agent Cryptophycin 52 (LY355703) Induces Apoptosis via Multiple Pathways in Human Prostate Cancer Cells[1]

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ABSTRACT

We assessed the ability of cryptophycin 52 (LY355703), a novel antimicrotubule, to induce growth arrest and apoptosis in prostate cancer cell lines and investigated potential molecular mechanisms of death. LNCaP (androgen-dependent) and DU-145 (androgen-independent) cells accumulated in G_2-M phase of the cell cycle and progressively acquired sub-G_0-G_1 DNA content after 48 h of exposure to cryptophycin 52 (1–10 pM). Induction of apoptosis was confirmed by DNA ladder formation and detection of cytoplasmic nucleosomes. PC-3 (androgen-independent) cells were less responsive to cryptophycin 52-induced death. Apoptosis was associated with proteolytic processing and activation of the caspase-3-like subfamily proteins caspase-3 and caspase-7 and cleavage of the caspase substrate poly(ADP-ribose) polymerase. The pan-caspase inhibitor BOC-Asp(OMe)-fluoromethylketone effectively reduced cryptophycin 52-induced caspase-3-like protease activity and apoptosis in DU-145 cells. In contrast, BOC-Asp(OMe)-fluoromethylketone did not inhibit apoptosis induction in LNCaP cells by cryptophycin 52, even though both cryptophycin 52-induced caspase-3-like activity and staurosporine-induced death were blocked under identical conditions. Cryptophycin 52 induced phosphorylation of c-raf1 and bcl-2 and/or bcl-x_L to comparable levels in all cell lines studied, and LNCaP cells overexpressing bcl-2 were more resistant to cryptophycin 52-induced apoptosis. Up-regulation of p53, bax, and p21 expression was induced in wild-type p53-expressing LNCaP cells only after cryptophycin 52 exposure. A sustained increase in c-Jun NH_2-terminal kinase phosphorylation was also observed, the levels of which strongly correlated with apoptosis. We conclude that apoptosis induced by cryptophycin 52 in prostate cancer cells is androgen status independent, cell type specific for caspase requirement, modulated by the bcl-2 family, linked to but not dependent on p53, and strongly correlated with c-Jun NH_2-terminal kinase phosphorylation. Cryptophycin 52-induced apoptosis in prostate cancer cells is therefore associated with multiple cell line-specific alterations in apoptosis-associated proteins and pathways.

INTRODUCTION

Androgen ablation is the standard of care for patients who present advanced, hormone-sensitive metastatic prostate cancer. Although this therapy typically results in rapid and dramatic regression of the prostate cancer, almost all patients progress and eventually die from hormone-refractory disease (1).

Antimicrotubule agents, including the taxanes (docetaxel and paclitaxel), *Vinca* alkaloids (vincristine, vinorelbine, and vinblastine), and estramustine phosphate, used either alone or in combination, have potent clinical activity in patients with hormone-refractory prostate cancer (2–4). Microtubule damage in cells treated *in vitro* with such drugs is associated with apoptosis and the induction of multiple apoptosis-related signaling pathways in different cell types (5). These include but are not limited to up-regulation of the tumor suppressor gene p53, apoptosis promoters bax and bak, and cyclin-dependent kinase inhibitor p21; phosphorylation of c-raf1 and the apoptosis suppressors bcl-2 and bcl-x_L; and down-regulation of bcl-x_L (6–13). Activation/inactivation of several protein kinases including Ras/Raf and the stress-activated kinase JNK[3] (14–16) and a requirement for the cysteine protease caspase cascade have also been reported (17–18). However, it is presently unclear which of these events can occur concurrently in response to microtubule damage and which are critical for the execution of cell death.

A new addition to the family of microtubule targeting agents is cryptophycin 52 (LY355703), a macrocyclic depsipeptide (Fig. 1). It is a synthetic analogue of naturally occurring cryptophycins isolated from the blue-green algae species *Nostoc* (19). Cryptophycin 52 binds to the *Vinca* domain of microtubules, inhibits microtubule polymerization, and is the most potent suppressor of microtubule dynamics studied to date (20, 21). It has potent antiproliferative and cytotoxic activity against

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[3] The abbreviations used are: JNK, c-Jun NH_2-terminal kinase; Z-YVAD-FMK, *N*-benzyloxycarbonyl-Tyr-Val-Ala-Asp(OMe)-fluoromethylketone; Z-DEVD-FMK, *N*-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone; B-D-FMK, BOC-Asp(OMe)-fluoromethylketone; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; TBST, Tris-buffered saline containing 0.1% Tween 20; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

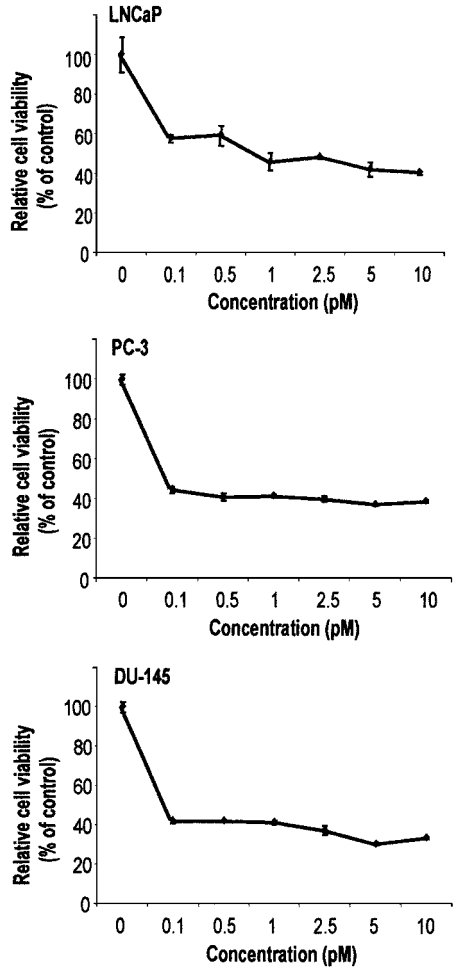

Fig. 1 Effect of cryptophycin 52 on prostate cancer cell viability. LNCaP, PC-3, and DU-145 cells were treated with DMSO (vehicle control) or the indicated concentration of cryptophycin 52 for 72 h. Cell viability was determined by MTT assay. Results represent relative cell viability (mean ± SD; experiments in triplicate) of DMSO control-treated cells where cell viability = 100%.

a broad spectrum of cultured human tumor cell lines, including several multidrug-resistant lines, and both murine solid tumors and human tumor xenografts in mice (22–27). It was demonstrated to be 40–400-fold more potent than paclitaxel and *Vinca* alkaloids and is currently undergoing clinical evaluation for efficacy in prostate, breast, and lung cancer. As observed with other antitubulin agents, the antiproliferative effect of cryptophycin 52 appears to be associated with its ability to block mitotic progression in cells (27). The cytotoxic effects appear to be mediated, at least in part, via apoptosis. However, the biochemical signals that lead to apoptosis in cryptophycin 52-treated cells have not been characterized.

We have examined the effects of cryptophycin 52 on cell viability, growth arrest, and apoptosis in the human prostate cancer cell lines LNCaP (wild-type p53, androgen dependent), PC-3 (null p53, androgen independent), and DU-145 (mutant p53, androgen independent; reviewed in Ref. 28). We also examined alterations in the expression and/or activity of a wide variety of apoptosis-associated proteins in all of these cell lines after cryptophycin 52 exposure and investigated the role of caspases in the execution of cell death

MATERIALS AND METHODS

Materials. Cryptophycin 52 was obtained from Eli Lilly and Company (Greenfield, IN). The pan-caspase inhibitor B-D-FMK was purchased from Enzyme Systems Products (Livermore, CA). The caspase-1-like (Z-YVAD-FMK) and caspase-3-like (Z-DEVD-FMK) inhibitors were from Alexis Biochemicals (San Diego, CA). Cryptophycin 52 and caspase inhibitors were dissolved in DMSO and stored in aliquots at –80°C. Primary antibodies anti-p53 (DO-1), anti-p21 (H-164), anti-bax (N-20), anti-bcl-x_L (H-5), anti-caspase-3 (E-8), and anti-PARP (F-2), used for Western blotting, were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-bcl-2 antibody was from Dako Corp. (Carpinteria, CA). Anti-JNK and anti-phospho-JNK antibodies were from New England Biolabs (Beverley, MA). Anti-caspase-7 antibody was from PharMingen (San Diego, CA).

Cell Culture. The human prostate cancer cell lines LNCaP (androgen dependent) and PC-3 and DU-145 (both androgen independent) were obtained from American Type Culture Collection (Manassas, VA). Bcl-2-overexpressing LNCaP cells (LN/Bcl-2-3) and neomycin-resistant controls cells (LN/neo) were a gift from Dr. A. Raffo (Columbia University, New York, NY; Ref. 29). Cells were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), 50 units/ml penicillin G, 50 μg/ml streptomycin, and 2 mM glutamine (Life Technologies, Inc.). LN/Bcl-2-3 and LN/neo cells were cultured in the same medium supplemented with 300 μg/ml G418 (Cellgro, Herndon, VA). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. For experiments, cells were trypsinized and allowed to adhere overnight before treatment in fresh medium for up to 48 h. All experiments were performed with cell lines in experimental growth phase. For caspase inhibition experiments, caspase inhibitors were added 20 min before the addition of cryptophycin 52. The final volume of DMSO in medium did not exceed 0.2% in any experiment.

Cytotoxicity. Cells were initially seeded at 3×10^3 cells in 96-well plates (Nunc Inc., Naperville, IL). Cells were allowed to adhere overnight and then treated with the indicated concentration of cryptophycin 52 for 72 h. The MTT assay was performed using the Cell Proliferation Kit 1 (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions.

PI Staining for DNA Content. Cells were initially seeded at 1×10^5 cells in 6-well dishes Cells were then incubated in the indicated concentration of cryptophycin 52 for up to 48 h. After treatment, adherent cells were gently scraped from the wells into the medium containing floating cells. Cells were then centrifuged, washed once in PBS, and resuspended in 200 μl of PBS. Cells were then added dropwise to 5 ml of ice-cold 70% ethanol with vortexing and stored at –20°C until analysis. Fixed cells were collected by centrifugation, washed once in PBS, and incubated in 300 μl of PI staining buffer [10

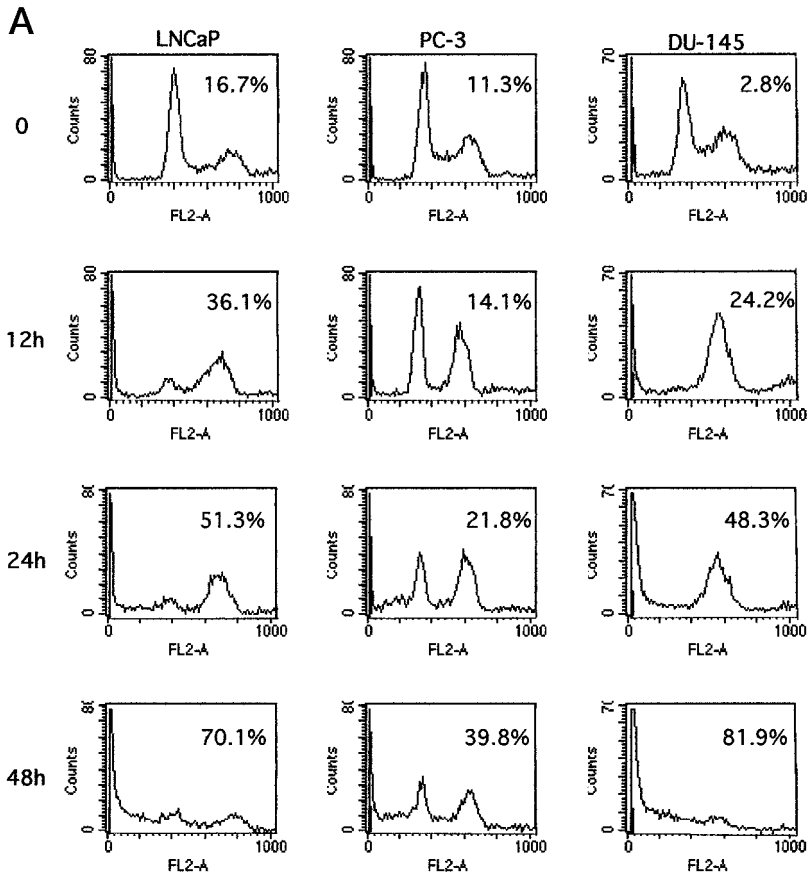

Fig. 2 Time and dose effect of cryptophycin 52 on cell cycle profile in prostate cancer cells. *A*, LNCaP, PC-3, and DU-145 cells were treated with 5 pM cryptophycin 52 for the indicated time and analyzed for PI-stained DNA content by flow cytometry. *B*, LNCaP, PC-3, and DU-145 cells were left untreated or treated with the indicated concentration of cryptophycin 52 for 48 h and analyzed for PI-stained DNA content by flow cytometry. Values indicate the percentage of cells with sub-G_0-G_1 DNA content. Three experiments were performed in triplicate.

μg/ml PI and 250 μg/ml RNase A (both from Sigma, St. Louis, MO) in PBS] for 30 min at room temperature. Samples were then acquired using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), and the sub-G_0-G_1 peak was quantified using CELLQuest software.

DNA Fragmentation Analysis by Gel Electrophoresis. DNA fragmentation assays were performed as described previously (12). Cells were initially seeded at 1.5×10^6 cells in 100-mm^2 dishes (Nunc Inc.). Cells were then incubated in the indicated concentration of cryptophycin 52 for up to 48 h. After treatment, adherent cells were gently scraped from the wells into the medium containing floating cells. Cells were then centrifuged, washed in PBS, and lysed in 300 μl of 0.5% Triton X-100, 20 mM EDTA, and 50 mM Tris-Cl (pH 7.5) at 4°C with rotation for 30 min. After centrifugation, 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. Proteinase K (final concentration, 2.5 mg/ml) was than added, and incubation at 56°C was continued for an additional 2 h. DNA was precipitated with 2.5 volumes of ethanol and dissolved in 10 mM Tris (pH 8) containing 1 mM EDTA. DNA was electrophoresed on 1.8% gels containing 0.5 mg/ml ethidium bromide and visualized by UV transillumination.

Determination of Cytoplasmic Mono- and Oligonucleosomes. Cells were initially seeded at 5×10^4 cells in 6-well dishes (Nunc Inc.) and then incubated in the indicated concentration of cryptophycin 52 for 48 h. The Cell Death Detection ELISA (Roche) was performed according to the manufacturer's instructions using cytosolic fractions obtained from pooled adherent and floating cells.

Western Blotting Analysis. Cells were initially seeded at 0.5×10^6 cells in 100-mm^2 dishes (Nunc Inc.). After treatment for up to 48 h with cryptophycin 52, adherent cells were gently scraped from the wells into the medium containing floating cells to obtain all cells. Cells were then centrifuged, washed in PBS, lysed in ice-cold lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1.5 mM EGTA, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 40 mM nitrophenylphosphate, and protease inhibitor mixture (50 μg/ml each of leupeptin, aprotinin, chymotrypsin, pepstatin A, and Peflabloc SC; all from Roche)], and cleared by microcentrifugation. Protein concentrations of lysates were determined using the Dc detergent-compatible assay (Bio-Rad, Hercules, CA), using BSA as a standard. Total cell lysates (50 μg) were separated by SDS-PAGE and electrophoretically transferred onto 0.45 μM nitrocellulose membranes (Micron Separations Inc., Keene, NH). Filters were blocked with 5% nonfat dry milk/TBST (or 5% BSA/TBST for bcl-2) and probed with appropriate dilutions (as recommended by the manufacturers) of primary antibody in 5% BSA/TBST overnight at 4°C. Membranes were then washed three times in TBST and subsequently incubated with appropriate horseradish peroxidase-conjugated secondary antibody

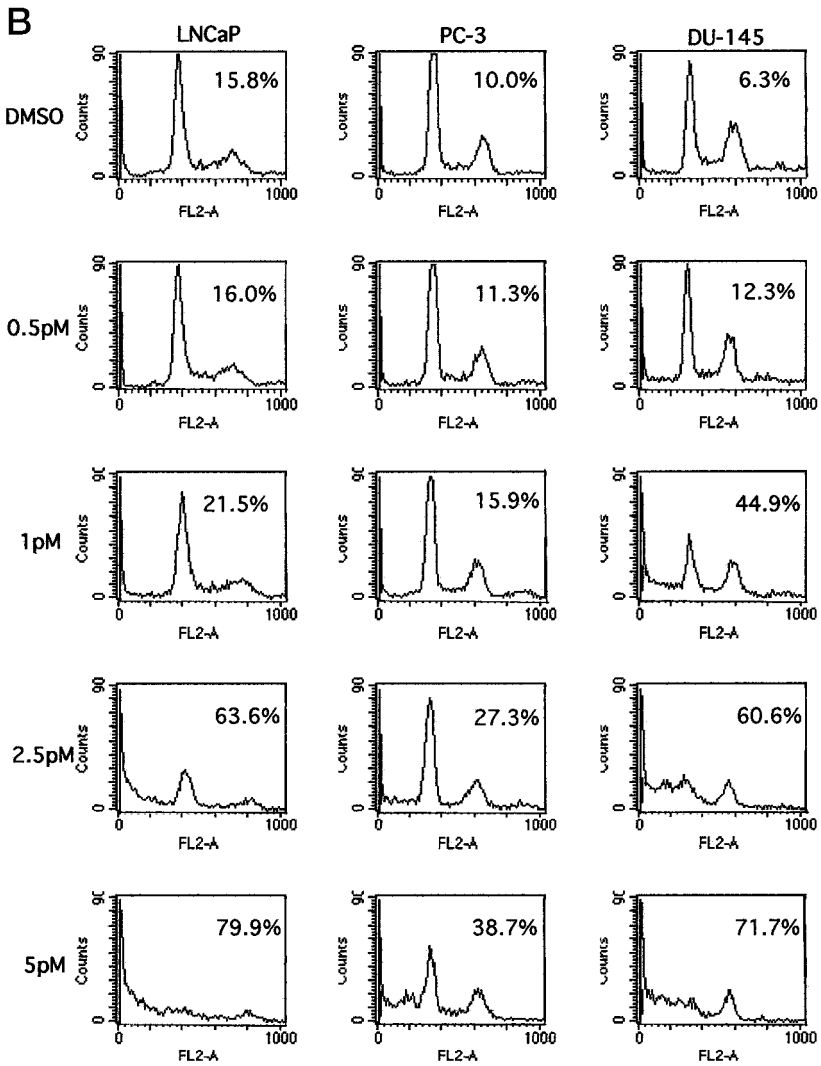

Fig. 2 Continued.

(Santa Cruz Biotechnology) for 1 h at room temperature in 5% nonfat dry milk/TBST. After washing, immunoreactive proteins were visualized using enhanced chemiluminescence (Amersham).

Caspase-3-like Protease Activity. Cells were initially seeded at 1×10^6 cells in 100-mm^2 dishes (Nunc Inc.). After treatment for 48 h with cryptophycin 52, adherent cells were gently scraped from the wells into the medium containing floating cells to obtain all cells. Cells were then centrifuged and washed in PBS. Caspase-3-like protease activity of cell lysates was determined using the ApoAlert caspase-3 colorimetric assay kit (Clontech, Palo Alto, CA) according to manufacturer's instructions.

RESULTS

Effect of Cryptophycin 52 on Prostate Cancer Cell Viability. Cryptophycin 52 at picomolar concentrations has potent antimitotic, antiproliferative, and cytotoxic activity in a variety of *in vitro* human tumor cell lines (27). The effect of this agent on prostate cancer cells was determined by MTT assay. As shown in Fig. 1, cryptophycin 52 induced a reduction in cell viability in LNCaP, PC-3, and DU-145 cells 72 h after treatment. Effects were observed in all three cell lines at concentrations of 0.1 pM and above. In DU-145 cells, viability at 72 h was 40% of DMSO-treated controls. In LNCaP and PC-3 cells, viability at 72 h was approximately 50% of DMSO-treated controls.

Time- and Dose-dependent Effects of Cryptophycin 52 on Growth Arrest and the Induction of Apoptosis in Prostate Cancer Cells. To further investigate the effects of cryptophycin 52 on cell cycle phases and apoptosis in prostate cancer, cells were treated for 0, 12, 24, or 48 h in medium containing varying concentrations of cryptophycin 52. DNA content was determined by flow cytometry of PI-stained cells. Fig. 2*A* shows the time effect on prostate cancer cells treated with 5 pM cryptophycin 52. By 12 h, there was an increased proportion of cells in G_2-M when compared with nontreated controls for all cell lines. An increase in the percentage of total

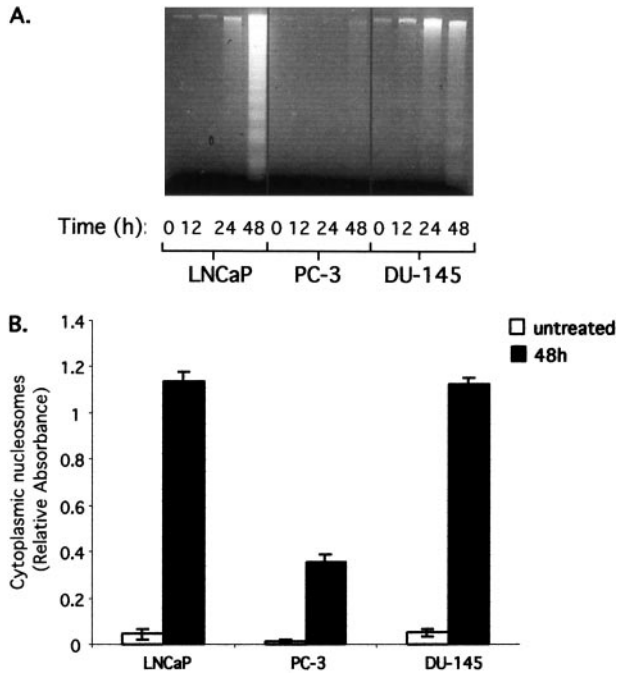

Fig. 3 Effect of cryptophycin 52 on DNA fragmentation in prostate cancer cells. *A*, LNCaP, PC-3, and DU-145 cells were treated with 5 pM cryptophycin 52 for the indicated times. Cytoplasmic DNA was isolated and electrophoresed on 1.8% gels containing ethidium bromide. *B*, LNCaP, PC-3, and DU-145 cells were left untreated or treated with 5 pM cryptophycin 52 for 48 h. Histone-associated DNA fragments in cytoplasmic fractions of cell lysates were determined using a photometric enzyme immunoassay. Results represent mean absorbance ± SD (experiments in triplicate).

cells in the sub-G_0-G_1 peak, characteristic of apoptotic cells, was observed progressively over 24- and 48-h exposure periods. PC-3 cells were less responsive to cryptophycin 52 than LNCaP and DU-145 cells with respect to G_2-M arrest over the same time frame. Formation of a sub-G_0-G_1 fraction was also reduced in PC-3 cells with this population derived from all cell cycle phases. This is in contrast to DU-145 and LNCaP cells, where the sub-G_0-G_1 peak was predominantly from the G_2-M phase. Similar time-dependent results were obtained for DU-145 cells treated for 12, 24, and 48 h with 50 nM paclitaxel (data not shown). Treatment of cells with 0.1% DMSO alone (vehicle control) for 48 h had no effect on cell cycle profile compared with untreated controls in any cell line tested (data not shown).

Fig. 2*B* shows the concentration effect of cryptophycin 52 on the cell cycle profile of prostate cancer cells treated for 48 h. A dose-dependent increase in the percentage of cells in the sub-G_0-G_1 peak was observed, starting at a concentration of 1 pM, in all prostate cancer cell lines studied as compared with untreated control cells.

To determine whether the cryptophycin 52-induced formation of a sub-G_0-G_1 peak was associated with apoptosis, DNA fragmentation analysis was performed. As shown in Fig. 3*A*, no ladder formation was observed in nontreated controls; however, a distinct DNA ladder typical of apoptosis was progressively observed in LNCaP and DU-145 cells treated with 5 pM cryptophycin 52 over a 48-h time period. PC-3 cells exposed to cryptophycin 52 under the same conditions showed less DNA ladder formation. Similar results were obtained for DU-145 cells treated for 48 h with 50 nM paclitaxel (data not shown). To further quantify DNA fragmentation, a cell death ELISA assay was performed on cells treated with 5 pM cryptophycin 52 to measure cytoplasmic nucleosome formation. As shown in Fig. 3*B*, cytoplasmic nucleosomes were increased over untreated control cells equally in LNCaP and DU-145 cells but less in PC-3 cells after treatment for 48 h. Similar results were also obtained for DU-145 cells treated for 48 h with 50 nM paclitaxel (data not shown).

Involvement of Caspases in Cryptophycin 52-induced Apoptosis in Prostate Cancer Cells. Caspases comprise three subfamilies: (*a*) an interleukin-1β-converting enzyme/caspase-1-like subfamily (caspases-1, -4 and -5); (*b*) a CPP32 (32-kDa cysteine protease)/caspase-3-like subfamily (caspases-3, -6, -7, -8, -9, and -10); and (*c*) an ICH/Nedd2 subfamily (caspase-2). Activation of caspases during apoptosis results in the cleavage and inactivation of a range of critical cellular substrates, including the DNA repair enzyme PARP (reviewed in Ref. 30).

Paclitaxel-induced death has been reported to be dependent on the activation of caspase-3-like as well as caspase-1-like proteases (17, 31). We therefore investigated the effect of cryptophycin 52 on the activation of the caspase-3-like subfamily. PARP is cleaved and inactivated by executioner caspase-3-like proteases from the 116-kDa full-length form to an inactive 85-kDa form. As shown in Fig. 4*A*, treatment of LNCaP cells with 5 pM cryptophycin 52 induced minimal proteolysis of PARP by 24 h, which was increased further, but not complete, by 48 h. In DU-145 cells, cleavage of full-length PARP to the apoptotic fragment was also observed by 24 h but was extensive and near completion by 48 h. PARP cleavage was also observed in DU-145 cells treated with 50 nM Taxol over 48 h (data not shown). In comparison, minimal cleavage of PARP was observed in PC-3 cells after a 48-h exposure to 5 pM cryptophycin 52.

The effect of cryptophycin 52 on cleavage of the inactive proenzymes to the active subunits of activated caspase-3 and -7 is shown in Fig. 4, *B* and *C*. Western blotting analysis revealed a time-dependent appearance of a weakly detected band around 17 kDa, representing the active subunit, when using an anti-caspase-3 antibody in both LNCaP and DU-145 cells after treatment with 5 pM cryptophycin 52 over 48 h (Fig. 4*B*). This band was less prominent in PC-3 cells treated under the same conditions. Generation of the active p20 large subunit form of caspase-7 was not detected by Western blotting in any cell line exposed to 5 pM cryptophycin 52 for up to 48 h (Fig. 4*C*). However, a p30 product was weakly detected by such treatment, which is probably due to removal of the prodomain from the protease before formation of the active p20, as reported previously for paclitaxel (31). These results suggest weak attenuated caspase-7 activation. In contrast, no cleavage of the upstream initiator caspase, caspase-8, was detected after cryptophycin 52 treatment (data not shown).

To confirm the formation of active caspase-3-like subunits as indicated by Western blotting, a caspase-3-like protease activity assay was performed. A 2.9-, 2.1-, and 3.5-fold increase in caspase-3-like protease activity over basal levels was observed in LNCaP, PC-3, and DU-145 cells, respectively, after a 48-h exposure to 5 pM cryptophycin 52 (Fig. 4*D*). An increase in

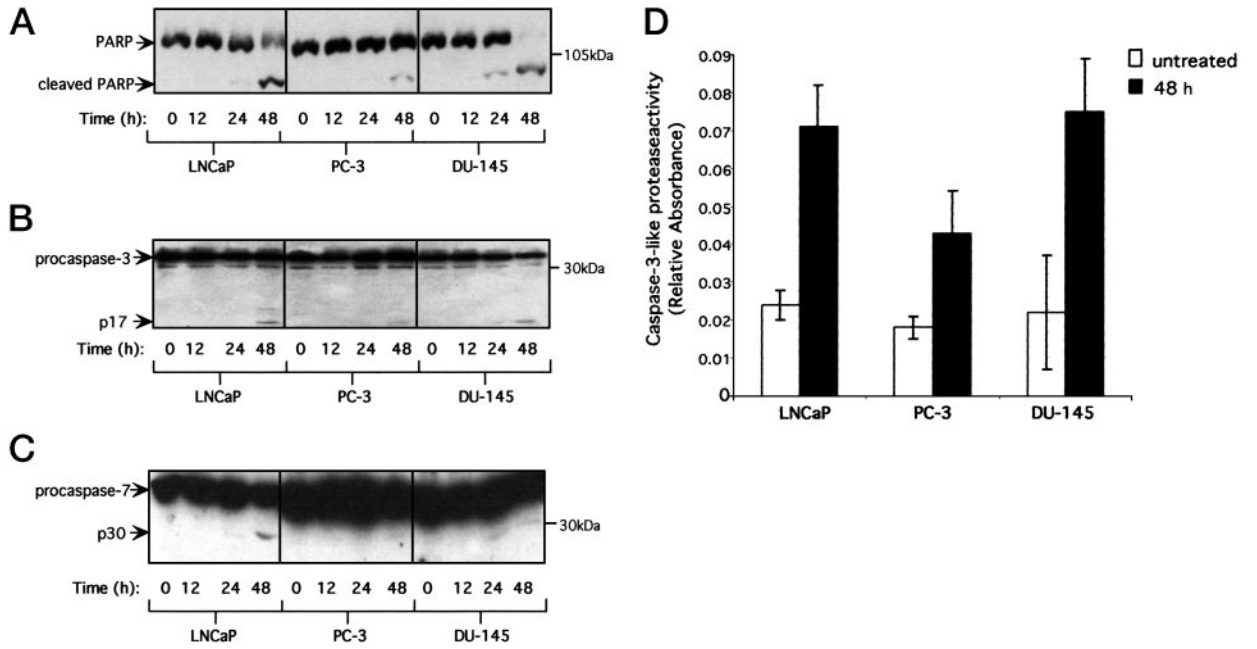

Fig. 4 Effect of cryptophycin 52 on PARP cleavage, caspase-3 and -7 cleavage, and caspase-3-like protease activity in prostate cancer cells. LNCaP, PC-3, and DU-145 cells were treated with 5 pM cryptophycin 52 for the indicated times. *A,* cell lysates were immunoblotted with an anti-PARP antibody. Cleavage of full-length PARP (116 kDa) to the apoptotic fragment (85 kDa) is indicated. *B,* cleavage of endogenous caspase-3 (proenzyme) to the active form (p17) was determined by immunoblotting of cell lysates with an anti-caspase-3 antibody. *C,* cell lystates were immunoblotted with an anti-caspase-7 antibody. Cleavage of caspase-7 proenzyme to the protease lacking the prodomain (p30) is indicated. The active form (p20) was not detected. *D,* caspase-3-like protease activity in cell lysates was assayed by spectrophometric detection of the chromophore *p*-nitrophenyl after cleavage from the labeled substrate DEVD-*p*-nitrophenyl. Results represent mean absorbance ± SD ($n = 3$).

caspase-3 activity was also observed in DU-145 cells treated over 48 h with 50 nM paclitaxel (data not shown).

Because cryptophycin 52 induced caspase-3-like proteolytic activity, we subsequently investigated whether or not caspase activation was essential for apoptosis to proceed. Therefore, LNCaP and DU-145 cells were preincubated with 50 μM B-D-FMK (pan-caspase inhibitor) for 20 min before 48-h exposure to 5 pM cryptophycin 52 to determine the possible involvement of caspases in cryptophycin 52-induced cell death. B-D-FMK completely reversed the induction of caspase-3-like protease activity (Fig. 5*A*) and significantly decreased PARP cleavage induced by cryptophycin 52 in LNCaP and DU-145 cells (Fig. 5*B*), thus verifying the ability of this compound at 50 μM to significantly inhibit caspase-mediated events in both lines. Flow cytometric analysis revealed that in the presence of B-D-FMK, the cryptophycin 52-induced formation of a sub-G_0-G_1 peak was significantly decreased in DU-145 cells, with cells accumulating in the G_2-M phase (Fig. 5*C*). In contrast, B-D-FMK had no effect on the cell cycle profile observed in LNCaP cells treated with cryptophycin 52. Identical results were obtained for DU-145 and LNCaP cells treated for 48 h with 100 nM paclitaxel in the presence or absence of 50 μM B-D-FMK (data not shown). However, 50 μM B-D-FMK treatment did inhibit staurosporine-induced sub-G_0-G_1 peak formation (4 μM, 16 h) in LNCaP cells (data not shown), confirming the ability of this inhibitor to prevent caspase-dependent apoptosis in this cell type. DNA fragmentation analysis by gel electrophoresis of LNCaP and DU-145 cells (Fig. 5*D*) confirmed caspase dependency for apoptosis of DU-145 cells, but not LNCaP cells, as indicated by the flow cytometric data. In addition, Z-YVAD-FMK (caspase-1-like inhibitor) and Z-DEVD-FMK (caspase-3-like inhibitor) were equally as effective as B-D-FMK in preventing apoptosis of DU-145 cells during cryptophycin 52 exposure (Fig. 6). Together, these results implicate the direct involvement of caspases, including caspase-1-like and caspase-3-like subfamilies, in cryptophycin 52-induced apoptosis in DU-145 cells, but not in LNCaP cells.

Modulation of Cell Cycle and Apoptosis-associated Proteins in Prostate Cancer Cells by Cryptophycin 52. To investigate additional and/or alternative mechanisms of cryptophycin 52-induced cell death besides caspases, the effect of this agent on p53, p21, bax, bcl-2, bcl-x_L, c-raf1, and JNK proteins was assessed. Cells were treated for 0, 12, 24, or 48 h with 5 pM cryptophycin 52, and protein levels were determined by Western blotting (Fig. 7). LNCaP, PC-3, and DU-145 cells have previously been characterized as having wild-type p53, null p53, and mutant p53, respectively (28).

The p53 protein level of LNCaP cells increased after exposure to cryptophycin 52 over 48 h, which was associated with an increase in the p53-regulated proteins p21 and bax. In contrast, p53 and p21 were not detected at any time in PC-3 cells, and bax levels remained unchanged. Furthermore, the high p53 level detected in DU-145 cells was not altered by cryptophycin 52 treatment, and p21 and bax were not detected at any time points tested.

Phosphorylation of bcl-2 and/or bcl-x_L can be detected as

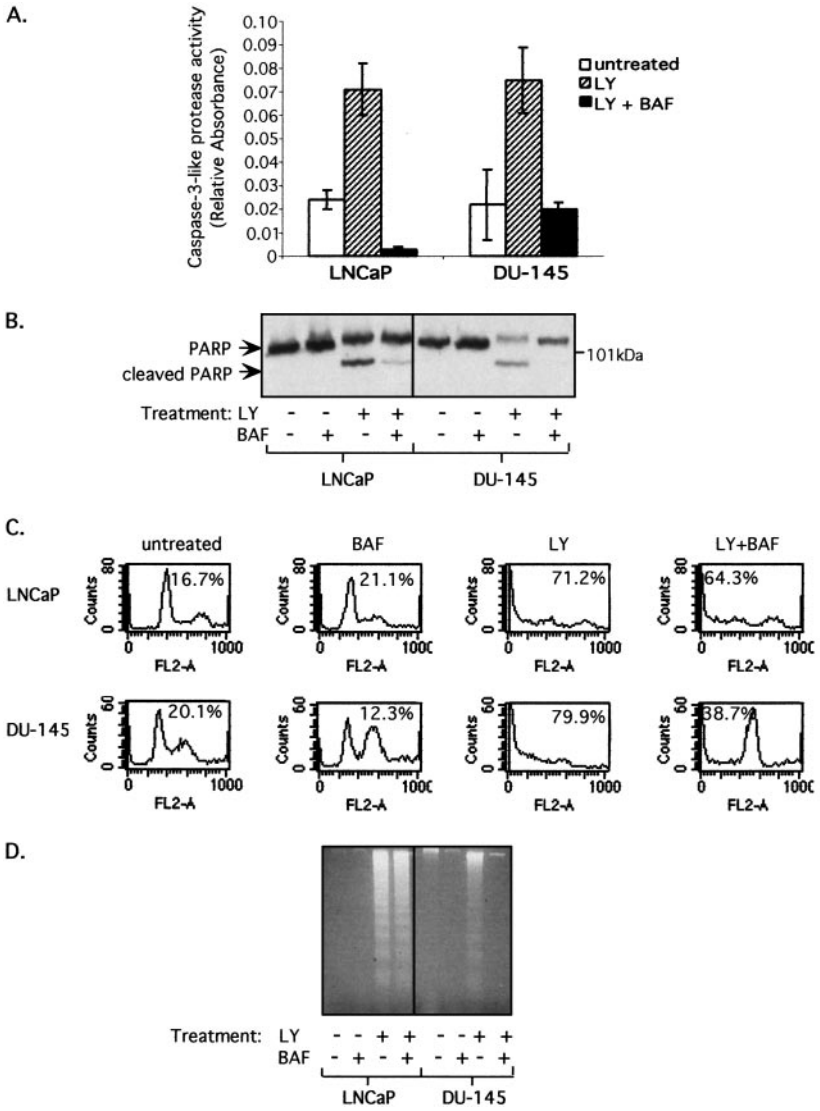

Fig. 5 Effect of caspase inhibition on cryptophycin 52-induced apoptosis in LNCaP and DU-145 cells. LNCaP and DU-145 cells were left untreated (−) or treated (+) with the pan-caspase inhibitor B-D-FMK (50 μM; *BAF*) for 20 min and incubated with or without 5 pM cryptophycin 52 (*LY*) for an additional 48 h. *A*, caspase-3 subfamily protease activity in cell lysates was determined as described in the Fig. 3 legend. *B*, cell lysates were immunoblotted with an anti-PARP antibody. The *arrow* indicates cleaved PARP (85 kDa). *C*, cells were analyzed for PI-stained DNA content by flow cytometry. Values indicate the percentage of cells with sub-G_0-G_1 DNA content. *D*, cytoplasmic DNA was isolated and electrophoresed on 1.8% gels containing ethidium bromide.

more slowly migrating bands on Western blots detected using anti-bcl-2 and anti-bcl-x_L antibodies (8, 9, 11, 12). Using this approach, we detected a mobility shift of bcl-2 in both LNCaP and PC-3 cells as early as 12 h, which increased further at 24 h and then decreased at 48 h after cryptophycin 52 treatment. bcl-x_L was detected as a doublet in all untreated prostate cancer lines studied and underwent a significant transient mobility shift in PC-3 and DU-145 cells after cryptophycin 52 exposure. In contrast, LNCaP cells did not show this mobility shift, and no loss of total bcl-x_L protein was observed in any cell line treated over 48 h. c-raf1 was also found to undergo a transient mobility shift in cryptophycin 52-treated cell extracts from all prostate cancer cell lines tested.

On activation, the stress-activated protein kinase JNK is phosphorylated (14). Phosphorylation of the two forms of JNK, p46 and p54, increased in response to cryptophycin 52 treatment as detected on Western blots with a phospho-specific antibody. This was observed only after 24 h of exposure and was further increased by 48 h in LNCaP and DU-145 cells without affecting total JNK protein expression. The increase in JNK phosphorylation in PC-3 cells, which had low total JNK protein levels and undetectable basal levels of phosphorylation, was minimal after 48 h of treatment.

Effect of bcl-2 Overexpression on Cryptophycin 52-induced Apoptosis in LNCaP Prostate Cancer Cells. To determine whether overexpression of bcl-2 in LNCaP cells altered the response to cryptophycin 52-induced apoptosis, we investigated the effect of 5 pM cryptophycin 52 on apoptosis at time 0, 12, 24, and 48 h on LN/Bcl-2-3 and LN/neo cell lines. Increased expression of bcl-2 in LN/Bcl-2-3 cells, as compared with LN/neo and LNCaP control cell lines, was confirmed by Western blotting (data not shown). The LN/Bcl-2-3 cell line was significantly less responsive than LN/neo cells to 5 pM cryptophycin 52 at 24 and 48 h with respect to DNA ladder formation (Fig. 8*A*), detection of cytoplasmic nucleosomes (Fig. 8*B*), and PARP cleavage (Fig. 8*C*).

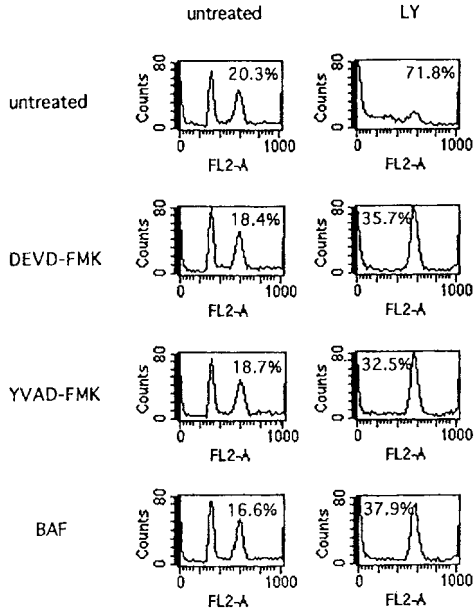

Fig. 6 Effect of inhibition of caspase-1-like and caspase-3-like proteases on cryptophycin 52-induced apoptosis in DU-145 cells. Cells were left untreated or treated with 20 μM Z-YVAD-FMK (*YVAD-FMK*; caspase-1-like inhibitor) or 20 μM Z-DEVD-FMK (*DEVD-FMK*; caspase-3-like inhibitor) with or without 5 pM cryptophycin 52 (*LY*) for 48 h. Cells were analyzed for PI-stained DNA content by flow cytometry. Values indicate the percentage of cells with sub-G_0-G_1 DNA content. The effect of the pan-caspase inhibitor B-D-FMK (50 μM; *BAF*) carried out at the same time is shown for comparison.

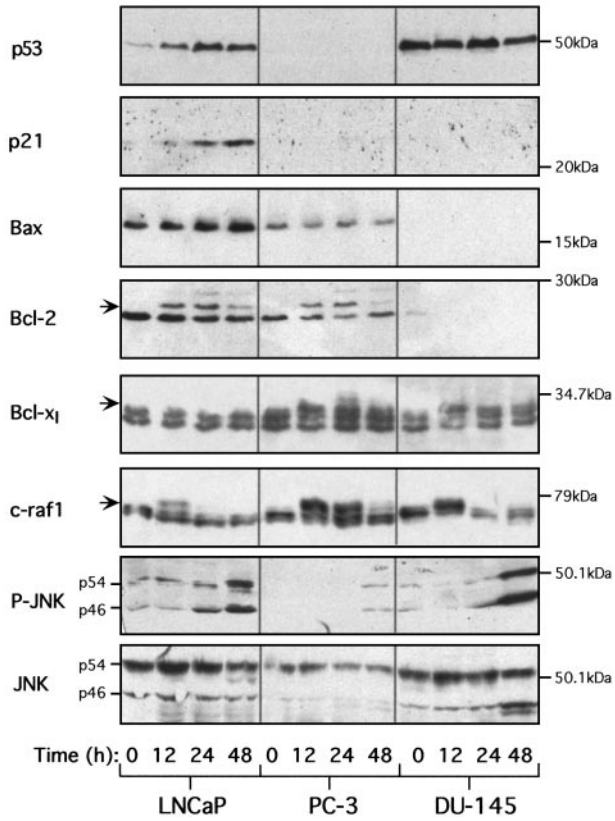

Fig. 7 Effect of cryptophycin 52 on cell cycle and apoptosis-related proteins in prostate cancer cells. LNCaP, PC-3, and DU-145 cells were treated with 5 pM cryptophycin 52 for the indicated times. Cell lysates were immunoblotted with antibodies against the indicated proteins. For bcl-2, bcl-x_L, and c-raf1, *arrows* indicate mobility shift representative of transient phosphorylation induced by cryptophycin 52 treatment.

DISCUSSION

The antimicrotubule cryptophycin 52 has been shown to have potent broad-spectrum antimitotic, antiproliferative, and cytotoxic activity against human lymphoma, leukemia, colon, cervical, and breast cancer cells *in vitro* (27). In this report, we extend these observations to prostate cancer lines.

Other cytotoxic antitubulin agents induce G_2-M arrest and subsequent apoptosis in a range of human cancer cells, including prostate cancer lines (10–12). Cryptophycin 52 also induced a time- and dose-dependent induction of G_2-M arrest and apoptosis in LNCaP and DU-145 cells. We therefore propose that the cytotoxic effects of this agent are also mediated, at least in part, via apoptosis, which is in agreement with a previous study (27). However, the picomolar concentrations required for cryptophycin 52-induced apoptosis were substantially lower than the nanomolar concentrations reported previously and observed by us for docetaxel- and paclitaxel-induced death in the same prostate cancer lines (12, 18, 31). Although DU-145 cells were previously characterized as being resistant to paclitaxel (11), we found this cell line to be responsive to both cryptophycin 52 and the taxanes. Similar to our findings for cryptophycin 52, a diminished apoptotic response of PC-3 cells as compared with LNCaP cells was observed for paclitaxel (12, 18).

Apoptosis induced by antitubulin agents has been associated with alterations in a variety of cellular signaling pathways. Caspase activation frequently plays a key role in apoptosis induced by a wide range of chemotherapeutic drugs. Paclitaxel-induced apoptosis in HeLa cells and osteosarcoma Saos-2 cells and docetaxel-induced cell death in mouse fibroblast L929 cells were blocked by caspase inhibition (10, 17, 31). Similarly, we observed a requirement for caspases in cryptophycin 52-induced death of DU-145 prostate cancer cells. Cryptophycin 52 induced caspase-3-like protease activity and extensive cleavage of PARP in this cell line. Furthermore, the caspase inhibitors B-D-FMK (pan-caspase inhibitor), DEVD-FMK (caspase-3-like inhibitor), and YVAD-FMK (caspase-1-like inhibitor) were equally effective in rescuing cells from cryptophycin 52-induced and paclitaxel- and docetaxel-induced apoptosis. These results suggest that both caspase-3-like and caspase-1-like protease activation are critical for death of DU-145 cells in response to microtubule damage. This dual subfamily caspase requirement was also reported for docetaxel-induced death of L929 cells (17).

Apoptosis may, in certain cases, proceed in a caspase-independent manner. For example, thapsigargin-induced apoptosis in the human prostate cancer cell line TSU-pr1 was associated with caspase-3-like protease activity, even though caspase inhibition did not prevent the initiation of apoptosis (32). Although paclitaxel has been reported to induce weak caspase-7 cleavage and partial PARP cleavage in LNCaP cells, the role of caspase activation was not studied (18). Indeed,

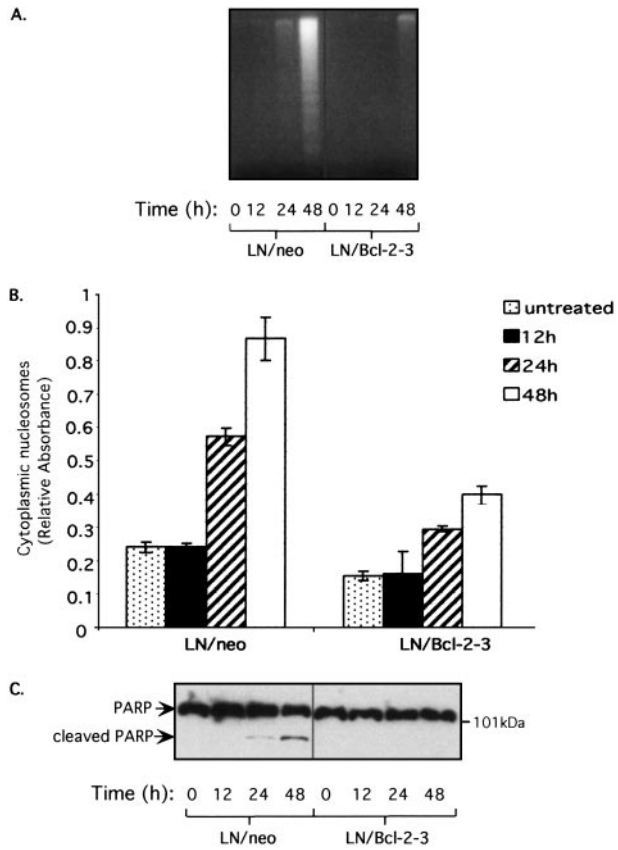

Fig. 8 Effect of bcl-2 overexpression on cryptophycin 52-induced apoptosis in LNCaP cells. LN/neo and LN/Bcl-2-3 cells were treated with 5 pM cryptophycin 52 for the indicated times. *A*, cytoplasmic DNA was isolated and electrophoresed on 1.8% gels containing ethidium bromide. *B*, histone-associated DNA fragments in cytoplasmic fractions of cell lysates were determined using a photometric enzyme immunoassay. Results represent mean absorbance ± SD, $n = 3$. *C*, cell lysates were immunoblotted with an anti-PARP antibody. The *arrow* indicates cleaved PARP (85 kDa).

although cryptophycin 52 induced caspase-3-like protease activity and partial PARP cleavage, caspase activation was not required for apoptosis of LNCaP cells. We also found that paclitaxel- and docetaxel-induced death in LNCaP cells was also caspase independent. Therefore, unlike previous reports in which lovastatin-, sodium phenylacetate-, and staurosporine-induced death was caspase dependent in LNCaP cells (33, 34), caspases are not a critical mediator of apoptosis associated with microtubule damage in this cell line. Together, these results suggest that activation of the caspase cascade may be associated with but not always essential for apoptosis to occur in response to microtubule damage. This requirement may depend on androgen sensitivity and/or other signaling pathways available and their regulation in a particular cell type.

Additional and/or alternative molecular mechanisms to caspases whereby cryptophycin 52 may induce apoptosis in prostate cancer cells were also investigated in our study. Phosphorylation of the bcl-2 and bcl-x_L proteins has been proposed to play a role in inhibiting their antiapoptotic function (8, 9, 35–37), and antitubulin agents have been reported to induce phosphorylation of c-raf1, bcl-2, and bcl-x_L in a range of cell lines (8, 10, 11, 18, 38). The concept is that c-raf1 activation and bcl-2/bcl-x_L phosphorylation represent early key steps in the cell death pathway induced by microtubule disruption. In accordance with these earlier findings, we found cryptophycin 52 to induce transient phosphorylation c-raf1 (LNCaP, PC-3, and DU-145), bcl-2 (LNCaP and PC-3), and bcl-x_L (PC-3 and DU-145) by 12 h as indicated by a mobility shift in Western blots. However, because PC-3 cells are less sensitive to cryptophycin 52 than the other cell lines, additional downstream death machinery must be required for apoptosis, which is perturbed in PC-3 cells. A similar conclusion was made for K562 cells compared with HL60 cells (31, 38). However, this would only be the case if modulation of bcl-2 and/or bcl-x_L is a critical mediator of apoptosis. It is possible that phosphorylation of bcl-2/bcl-x_L may just represent M phase events and may not be a mechanistic determinant of apoptosis (39).

In addition to altered activity, alterations in the levels of pro- and/or antiapoptotic proteins may also promote apoptosis. Paclitaxel increased bax expression in wild-type p53 human ovarian carcinoma cells (7). Similarly, cryptophycin 52 induced up-regulation of p53 protein and the p53-regulated genes p21 and bax in LNCaP cells with wild-type p53. However, our results suggest that antimicrotubule-induced apoptosis in prostate cancer cells is not strictly dependent on wild-type p53 because mutant p53 DU-145 cells were equally responsive to cryptophycin 52 as LNCaP cells. Even so, we cannot yet rule out the possibility that p53 is required for the cryptophycin 52-induced, caspase-independent death of LNCaP cells. Indeed, bax induced a caspase-independent cell death pathway in the pheochromocytoma cell line PC-12 (40). In contrast to our observations for LNCaP cells exposed to cryptophycin 52 in which bcl-x_L levels remained unaltered, treatment of the same cell type with paclitaxel led to a total down-regulation of bcl-x_L (12). It is unclear why these observations are inconsistent, but they may reflect drug-specific effects. Elevated expression of bcl-2 in LNCaP cells was highly protective against serum starvation- and phorbol ester-induced apoptosis (29), and overexpression of bcl-2 or bcl-x_L also protected HL60 cells from paclitaxel-induced PARP cleavage and apoptosis (41). We extend these results to show that bcl-2 overexpression in LNCaP cells suppressed cryptophycin 52-induced, caspase-independent apoptosis. Overexpression of bcl-2 has also previously been reported to have a cytoprotective function against caspase-independent apoptosis induced by vitamin D and bax overexpression (40, 42).

JNK is activated by microtubule inhibitors in a variety of human cell lines, suggesting that this may be a general stress response to microtubule dysfunction (14–16 43, 44). We also observed a sustained increase in JNK phosphorylation in prostate cancer cell lines treated with cryptophycin 52 that correlated with the apoptotic response. PC-3 cells have also previously been reported not to undergo apoptosis or JNK activation in response to *N*-(4-hydroxyphenyl)retinamide due to defects upstream of the JNK pathway (45). Although JNK activation may regulate the cell cycle and is important for apoptosis (46), the possibility that activation of JNK in our system is a stress response secondary to apoptosis cannot be ruled out. We are

therefore currently investigating the role of JNK as another mediator of cryptophycin 52-induced apoptosis in prostate cancer cells using a dominant negative approach. This pathway may represent a potential pathway associated with caspase-dependent apoptosis or for inducing caspase-independent death after microtubule damage.

In summary, we have demonstrated the recently described antitubulin agent cryptophycin 52 to be a potent and effective inducer of apoptosis at picomolar concentrations in the prostate cancer cell lines LNCaP and DU-145. A clinical trial to evaluate the efficacy of cryptophycin 52 in patients with hormone-refractory prostate cancer is currently under way (D. Petrylak). Apoptosis induced by cryptophycin 52 was caspase-3-like and caspase-1-like dependent or caspase independent, according to the prostate cancer cell line studied. Death was also associated with but not solely dependent on the phosphorylation of bcl-2 family members in all cell lines studied and inhibited by increased bcl-2 levels. Furthermore, cryptophycin 52-mediated death appeared to be androgen and p53 status independent and strongly correlated with JNK phosphorylation in prostate cancer cells. We therefore propose that apoptosis resulting from microtubule damage can proceed through multiple mechanisms, and the pathways used depend on the nature of the cell lines studied.

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