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26S Proteasome Inhibition Induces Apoptosis and Limits Growth of Human Pancreatic Cancer

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Abstract The 26S proteasome degrades proteins that regulate transcription factor activation, cell cycle progression, and apoptosis. In cancer, this may allow for uncontrolled cell division, promoting tumor growth, and spread. We examined whether selective inhibition of the 26S proteasome with PS-341, a dipeptide boronic acid analogue, would block proliferation and induce apoptosis in human pancreatic cancer. Proteasome inhibition significantly blocked mitogen (FCS) induced proliferation of BxPC3 human pancreatic cancer cells in vitro, while arresting cell cycle progression and inducing apoptosis by 24 h. Accumulation of $p21^{Cip1\text{-}Waf\text{-}1}$, a cyclin dependent kinase (CDK) inhibitor normally degraded by the 26S proteasome, occurred by 3 h and correlated with cell cycle arrest. When BxPC3 pancreatic cancer xenografts were established in athymic nu/nu mice, weekly administration of 1 mg/kg PS-341 significantly inhibited tumor growth. Both cellular apoptosis and $p21^{Cip1\text{-}Waf\text{-}1}$ protein levels were increased in PS-341 treated xenografts. Inhibition of tumor xenograft growth was greatest (89%) when PS-341 was combined with the tumoricidal agent CPT-11. Combined CPT-11/PS-341 therapy, but not single agent therapy, yielded highly apoptotic tumors, significantly inhibited tumor cell proliferation, and blocked NF-κB activation indicating this systemic therapy was effective at the cancer cell level. 26S proteasome inhibition may represent a new therapeutic approach against this highly resistant and lethal malignancy. J. Cell. Biochem. 82: 110–122, 2001.

Key words: cell cycle; p21; mouse xenografts; PS-341; NF-κB

Pancreatic cancer is one of the most lethal malignancies worldwide. In America, 28,000 new cases are expected to occur in 2001. Despite perceived advances in aggressive surgical and adjuvant therapies, clinicians are at a stalemate with 5-year survival rates of only 3–5% [Greenlee et al., 2000]. Novel biological and chemotherapies that control other tumors fail in pancreatic cancer. For outcomes to improve, new treatment strategies are needed.

Tumor growth in human pancreatic adenocarcinoma is associated with genetic alterations of cell cycle regulators (oncogenes and tumor suppressors) and abnormal responses to extracellular proteins (growth factors and cytokines) [Perugini et al., 1998]. Altered degradation of cell cycle proteins (p21, p27, p53, and cyclins) can result in accelerated and uncontrolled cell division, promoting cancer growth and spread [Sherr, 1996]. In fact, some have correlated expression of such oncogenes with clinical prognosis in pancreatic cancer [Bartsch et al., 1996; Lu et al., 1999]. These mutations also regulate apoptosis, or programmed cell death. Since the balance of apoptosis and cell cycle progression ultimately determines tumor growth, new therapies can succeed by targeting these cellular events.

Ubiquitin-proteasome degradation of intracellular proteins is highly regulated in eukaryotic cells. For example, this pathway regulates the processing of MHC class I-restricted antigens [Rock et al., 1994], and metabolic enzymes such as tyrosine amino transferase (TAT) and Cu/Zn superoxide dismutase [Hoffman et al., 1996; Gross-Mesilaty et al., 1997]. Several cell cycle protein substrates are also degraded by

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the proteasome including p21, p27, p53, and cyclins [Scheffner et al., 1990; Glotzer et al., 1991; Pagano et al., 1995].

Ubiquitin-proteasome degradation is also required for the processing of the NF-κB transcription factor whose inhibitory cytoplasmic protein, IκB, is phosphorylated and rapidly degraded by the proteasome complex. The degradation of IκB allows for the translocation of the unbound NF-κB to the nucleus [Palombella et al., 1994; Griscavage et al., 1996; Rousset et al., 1996], where the transcription of anti-apoptotic proteins such as IAP-1 and IAP-2 occurs [Wang et al., 1998]. Stabilization of IκB blocks NF-κB activation and increases cell susceptibility to apoptosis [Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996]. Since targeted ubiquitin-proteasome inhibition can stabilize IκB [McDade et al., 1999], apoptosis may increase in cancer and limit tumor growth.

Recently, highly selective inhibitors of the 26S proteasome have been developed and tested against common human malignancies including breast [Teicher et al., 1999] and prostate [Adams et al., 1999] cancer. Prompted by encouraging experimental results, PS-341, a dipeptide boronic acid inhibitor, is currently being evaluated in Phase I toxicity studies. We report that 26S proteasome inhibition results in decreased proliferation and increased apoptosis in vitro and correlates with significantly reduced tumor growth in vivo in human pancreatic cancer.

MATERIALS AND METHODS

Cell Culture and Treatments

The BxPC3 human pancreatic adenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown and propagated in RPMI supplemented with 10% fetal calf serum (FCS), 1% penicillin and streptomycin, and maintained at 37°C and 5% CO_2 atmosphere. PS-341 (Millennium Pharmaceuticals Inc, Cambridge, MA), a dipeptide boronic acid inhibitor, was prepared in 0.9% w/v sterile saline solution with 0.1% w/v ascorbic acid (vehicle) and stored at 4°C. CPT-11 (Camptosar® Pharmacia & Upjohn, Peapack, NJ) was reconstituted in D_5W to a stock concentration of 20 mg/ml and stored at room temperature. Human recombinant TNF-α (Genzyme, Cambridge, MA) was used at a concentration of 400 U/ml (specific gravity 5.88×10^4 U/mg). All in vitro assays were carried out in quadruplicate. Following serum starvation for 60 h to induce quiescence, cells were stimulated to proliferate by re-supplementation with media containing mitogen (10% FCS) alone or in combination with PS-341 (10 or 100 nM).

Proliferation Assays

BxPC3 cell proliferation was first determined by MTT assay. 2×10^4 cells were placed in phenol-free DMEM with 0.1 mg/ml of MTT [(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, (Sigma, St. Louis)] and incubated at 37°C, 5% CO_2 for 3 h. The media was aspirated and the formazan crystals were dissolved in 100 μl Dimethyl Sulfoxide (DMSO). An MRX microplate reader measured absorbance at 570 nm, with a reference of 750 nm.

Proliferation was also measured using the BrDU (5-bromo-2′-deoxyuridine) cell proliferation assay (Roche, Indianapolis IN) designed to measure incorporation of the BrDU pyrimidine analogue. A MRX microplate reader determined absorbance at 490 nm, with a reference of 750 nm. Proliferation in both assays was defined in relation to that of the quiescent serum starved cells prior to treatments by the following equation:

$$\text{Proliferation (\% control)} = \frac{\text{Absorbance treated sample}}{\text{Absorbance of cells after 60 h serum starvation}} \times 100$$

Cell Cycle Progression and Apoptosis

1×10^6 cells were plated in parallel in 25 cm^2 culture flasks. After serum starvation for 60 h, quiescent cells were replated with 10% FCS with or without PS-341. Cells were harvested daily by trypsinization, with care to include any floating cells, and then suspended in Phosphate Buffered Saline (PBS). They were then fixed with 70% ethanol and stored at −20°C. After 72 h, the ethanol was aspirated and cells were treated with propidium iodide (PI) at room temperature in darkness for 30 min. DNA histograms were obtained by fluorescence-activated cell sorting analysis (FACS). Cell cycle analysis was performed using the Modfit Program (Verity, Maine). Apoptosis was measured by quantifying the sub-G_0 peak, while cell cycle

progression was measured with corresponding absorbances for G_0-G_1, S, and G_2-M phases.

Apoptosis

Morphology. To verify further cell apoptosis, 1×10^6 cells were plated in parallel in 25 cm^2 culture flasks. After serum starvation for 60 h, quiescent cells were repleted with 10% FCS, with or without PS-341. After 24 and 48 h, cells were harvested with trypsin, suspended in 1 ml of PBS, and fixed with Pap stain. Morphologic evidence of apoptosis, including cell blebbing, chromatin condensation, and nuclear debris, were examined.

Immunoblotting

After specified treatments, cells were lysed by adding ice-cold RIPA buffer (1% NP-40, 50 mmol/L Tris, 150 mmol/L NaCl, 0.25% deoxycholate, 1 mmol/L EGTA, 1 mol/L NaF, and added protease inhibitor cocktail [Sigma, St. Louis, MO]). Equal protein loading was verified by Lowry protein quantification assay (Bio-Rad, Hercules, CA). Samples were solubilized for 5 min at 100°C in Laemmli's SDS-PAGE sample buffer, loaded onto a sodium dodecylsulfate-poly-acrylamide gel (SDS-PAGE) and electrophoresed. The gel was transferred to an Imobilon-P membrane (Millipore, Bedford, MA) at 105 V for 65 min. After incubation overnight at 4°C with 1:1,000 primary antibody dilution buffer (5% milk with 0.05% TTBS), membranes were developed using a 1:2,000 secondary antibody dilution buffer by enhanced chemiluminescence (New England BioLabs, Beverly, MA). Immunoblots were analyzed using primary antibodies for $p21^{Cip1\text{-}Waf\text{-}1}$ (Upstate Biotechnology, Lake Placid, NY), caspase-3 (Upstate Biotechnology, Lake Placid, NY), IkB-α (Cell Signaling Technology, Beverly, MA), or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

Electromobility Shift Assay of NF-κB

[γ-^{32}P]ATP was used to label a commercially available NF-κB double-stranded consensus oligonucleotide probe [Promega, Madison WI]. Briefly, 3.5 ρmole of oligonucleotide and 10 μCi of [γ-^{32}P]ATP were incubated with T4 polynucleotide kinase (1 U) at 37°C, then purified by column chromatography [Biorad] to yield 50,000 cpm/μl.

The binding reaction was modified from that of Sen and Baltimore [1986]. Ten microgram nuclear protein extract were incubated in a buffer containing 20 mM HEPES, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 200 μg/ml BSA, and 5% glycerol, with 2 μg poly dI/dC and 1 μl radiolabeled oligonucleotide probe. The binding reaction was incubated for 30 min at room temperature, then run on 6% polyacrylamide gels with 0.25X TBE (22 mM Tris-HCl, 22 mM boric acid, and 0.5 mM EDTA) on a minigel apparatus. Autoradiographic films were exposed to the gels for 24 h at −70°C and then developed.

Animals

6–8 week old athymic nu/nu mice were obtained (Harlan Sprague Dawley, St. Louis, MO) for tumor implantation. All animals were maintained in a sterile environment and cared for within the regulations of the Animal Medicine Facility at the University of Massachusetts Medical School (Animal Protocol A1050). The food, water, and bedding for these immunocompromised mice were sterilized and changed at least once weekly. Animals were acclimatized for two weeks before any manipulation.

Tumor Growth and Treatments

1×10^6 BxPC3 cells collected in 150 μl RPMI media in log phase growth were injected subcutaneously in the backs of 20 g athymic nu/nu mice 6–8 weeks old. Once tumor masses became established and palpable (50 mm^3), animals were randomized to receive four weekly intravenous (IV) or intraperitoneal (IP) injections of either vehicle (0.9% w/v sterile saline solution with 0.1% w/v ascorbic acid) or 1.0 mg/kg PS-341. In a second in vivo xenograft study, PS-341 was combined with the tumoricidal agent CPT-11. Once tumor masses became established, animals received either vehicle, IV CPT-11 (33 μg/kg) alone, IP PS-341 (1 mg/kg) alone, or IV CPT-11 (33 μg/kg) and IP PS-341 (1 mg/kg) in combination every four days for five treatments. Tumor volumes and body weight were measured biweekly. Tumor volume was measured along the longest orthogonal axes and calculated using the formula

$$\text{Volume} = \frac{\text{length} \times \text{width}^2}{2}$$

where width was the shortest measurement. Relative tumor volume was calculated as tumor volume on the day measured divided by initial tumor volume (V_x/V_i).

Tumor Xenograft Analysis

Tumor growth was measured for 40 days after the initiation of treatment. Next, animals were euthanized and tumors were harvested and stored for analysis. Tumors were sliced in 4 mm pieces and fixed in 10% formalin and embedded in paraffin blocks. Apoptosis was determined histologically using the TUNEL assay (ApopTag Peroxidase In Situ Kit, Intergen Co., Purchase, NY) in which nuclei staining orange were considered TUNEL positive (apoptotic). Proliferation was qualitatively examined using 5-Bromo-2′-deoxy-uridine labeling and detection kit II (Boehringer Mannheim, Indianapolis, IN). One hour after intratumoral BrDU injection, tumors were harvested and placed in 10% formalin. Cells proliferating and in S-phase incorporating BrDU into DNA stain red.

The remaining tumor samples were harvested, weighed, and immediately snap frozen in liquid nitrogen and stored at -70°C. They were then processed for protein analysis by the methods of Schneider [Schneider et al., 1986]. Tumor protein levels of $p21^{Cip1\text{-}Waf\text{-}1}$ (Upstate Biotechnology, Lake Placid, NY) were determined by immunoblotting. Membranes were reprobed for β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) to ensure equal protein loading.

Statistical Analysis

All in vitro data were analyzed with a two-tailed t-test with $P < 0.05$ considered significant. For in vivo analysis, effects of treatments and time were evaluated using analysis of variance (ANOVA) for repeated measures. When these effects were significant, pairwise differences were evaluated using Tukey's HSD (Honestly Significant Difference) to compensate for the additive Type I error due to multiple comparisons. Analyses were performed using the SPSS Version 10.0 statistical software package [Winer, 1971].

RESULTS

26S Proteasome Inhibition Inhibits Proliferation

BxPC3 cells were serum starved for 60 h to induce quiescence and then exposed to 10% FCS, with or without 10 or 100 nM PS-341. MTT analysis (Fig. 1A) revealed five-fold greater proliferation of cancer cells stimulated with

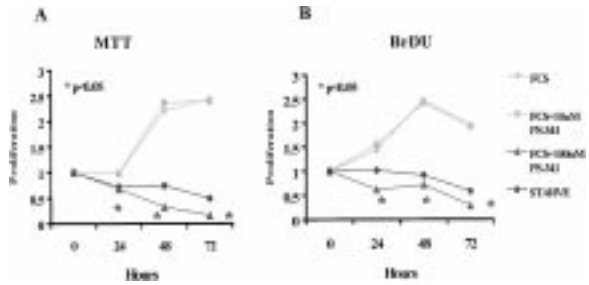

Fig. 1. BxPC3 Cell Proliferation Assays. Proliferation was measured with MTT assay (1A) and BrDU incorporation (1B). 2×10^4 BxPC3 cells were serum starved for 60 h to induce quiescence. They were then stimulated to proliferate with mitogen (10% FCS) with or without 10 or 100 nM PS-341. Results with 1,000 nM PS-341 are not shown. Standard errors are omitted for clarity. Results are representative of four similar experiments.

mitogen (10% FCS) for 72 h compared to those which did not proliferate when cultured in serum free media (RPMI alone). 100 nM PS-341 significantly ($P < 0.05$) inhibited BxPC3 cancer cell proliferation despite 10% FCS mitogen stimulation though 72 h. This effect was dose-dependent as a lower 10 nM PS-341 dose had no effect on mitogen-induced proliferation, while 1,000 nM was cytotoxic to all cells (not shown). BrDU incorporation (Fig. 1B), also used to assess cancer cell proliferation, showed identical significant inhibition of growth for BxPC3 cells treated with sufficient (100 nM) proteasome inhibitor.

PS-341 Induces Cell Cycle Arrest

PI staining and FACS analysis were used to determine any effects of 26S proteasome inhibition on mitogen-stimulated BxPC3 cell cycle progression (Fig. 2). After 12 h stimulation with 10% FCS, cells progressed through the cell cycle with only 37.4% identified in G_0-G_1 phase, and 43% identified in S phase. Proteasome inhibition with 100 nM PS-341 significantly blocked mitogen-induced cell cycle progression arresting 81.5% of cells in G_0-G_1 phase, with only 6.7% of cells progressing to S phase.

Induction of Apoptosis In Vitro

Treatment of BxPC3 human pancreatic cancer cells with 100 nM PS-341 increased apoptosis in a time dependent fashion. Apoptosis was significantly ($P < 0.05$) increased with 100 nM PS-341 as early as 24 h as demonstrated by apoptotic fractions of the FACS sub-G_0 peak (Fig. 3). At 48 and 72 h, apoptosis was 9.3 and

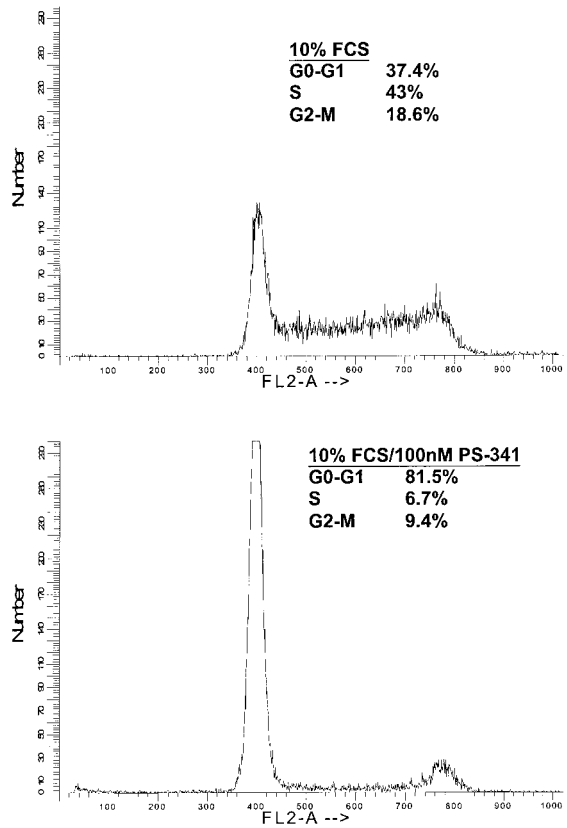

Fig. 2. 26S proteasome inhibition induces cell cycle arrest. 1×10^6 BxPC3 pancreatic cancer cells serum starved for 60 h to induce quiescence, and then restimulated to proliferate by 10% FCS with or without 10 or 100 nM PS-341 for 12 h. Cell cycle analysis was performed after PI staining of the cells using the Modfit LT program. Results are representative of three similar experiments.

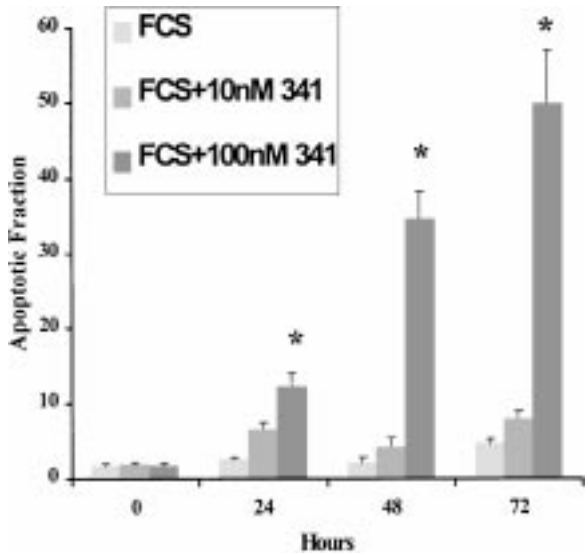

Fig. 3. 26S Proteasome inhibition induces apoptosis. FACS analysis of BxPC3 cells treated with PS-341. 1×10^6 quiescent cells were stimulated to proliferate (10% FCS) with or without 10 or 100 nM PS-341. Analysis of the sub-G_0 peak was performed after PI staining and use of the Modfit LT program. Apoptotic cell fraction is defined as the percentage of cells in the sub-G_0 phase of the cell cycle in each sample. Results are representative of four similar experiments.

11.4 times greater with 100 nM PS-341 than with FCS alone. 10 nM PS-341 failed to increase apoptosis significantly.

Cells were also examined histologically for evidence of apoptosis (Fig. 4). Cell blebbing, chromatin breakdown, and cellular debris, all morphologic characteristics of apoptosis, are evident within 24 h (Fig. 4B) in cells treated with the proteasome inhibitor. BxPC3 cells cultured in FCS without PS-341 did not show these apoptotic changes, instead maintaining normal cell structure (Fig. 4A). Further incubation for 48 h (Fig. 4C) with PS-341 appeared to potentiate apoptosis further. This morphologic evidence proves that observed changes in proliferation are due to apoptosis and not necrosis. This correlates with our FACS data demonstrating apoptosis to increase significantly from 24 to 48 h.

Caspase-3 activation, as occurs during apoptosis, is evident by 12 h (Fig. 5A) in PS-341 treated cells as indicated by cleavage of the inactive 32 kDa fragment to its active 17 kDa fragment. Caspase-3 activation is also dose-dependant (Fig. 5B) occurring after 24 h in cells treated with 100 nM to 10 μM of PS-341, correlating with the time course shown.

Through its interactions with cyclin dependent kinase complexes, $p21^{Cip1\text{-}Waf\text{-}1}$ retards cell cycle progression. $p21^{Cip1\text{-}Waf\text{-}1}$ must be degraded by the proteasome complex [Scheffner et al., 1990; Glotzer et al., 1991; Pagano et al., 1995] for cells to progress through the cell cycle. Accumulation of $p21^{Cip1\text{-}Waf\text{-}1}$ (Fig. 5C) is evident by 3 h with 100 nM PS-341 proteasome inhibition, consistent with the cell cycle arrest demonstrated by FACS analysis (Fig. 2). This effect, similar to BxPC3 cell proliferation, was dose dependent as $p21^{Cip1\text{-}Waf\text{-}1}$ accumulation did not occur with 10 nM PS-341 (not shown).

Inhibition of BxPC3 Tumor Xenograft Growth

To determine if systemic therapy with this 26S proteasome inhibitor could stunt tumor growth in animals, we established BxPC3 human pancreatic cancer xenografts in nu/nu athymic mice. To mimic a clinical scenario, we allowed tumors to become established and palpable (50 mm^3) before any treatments. Mice

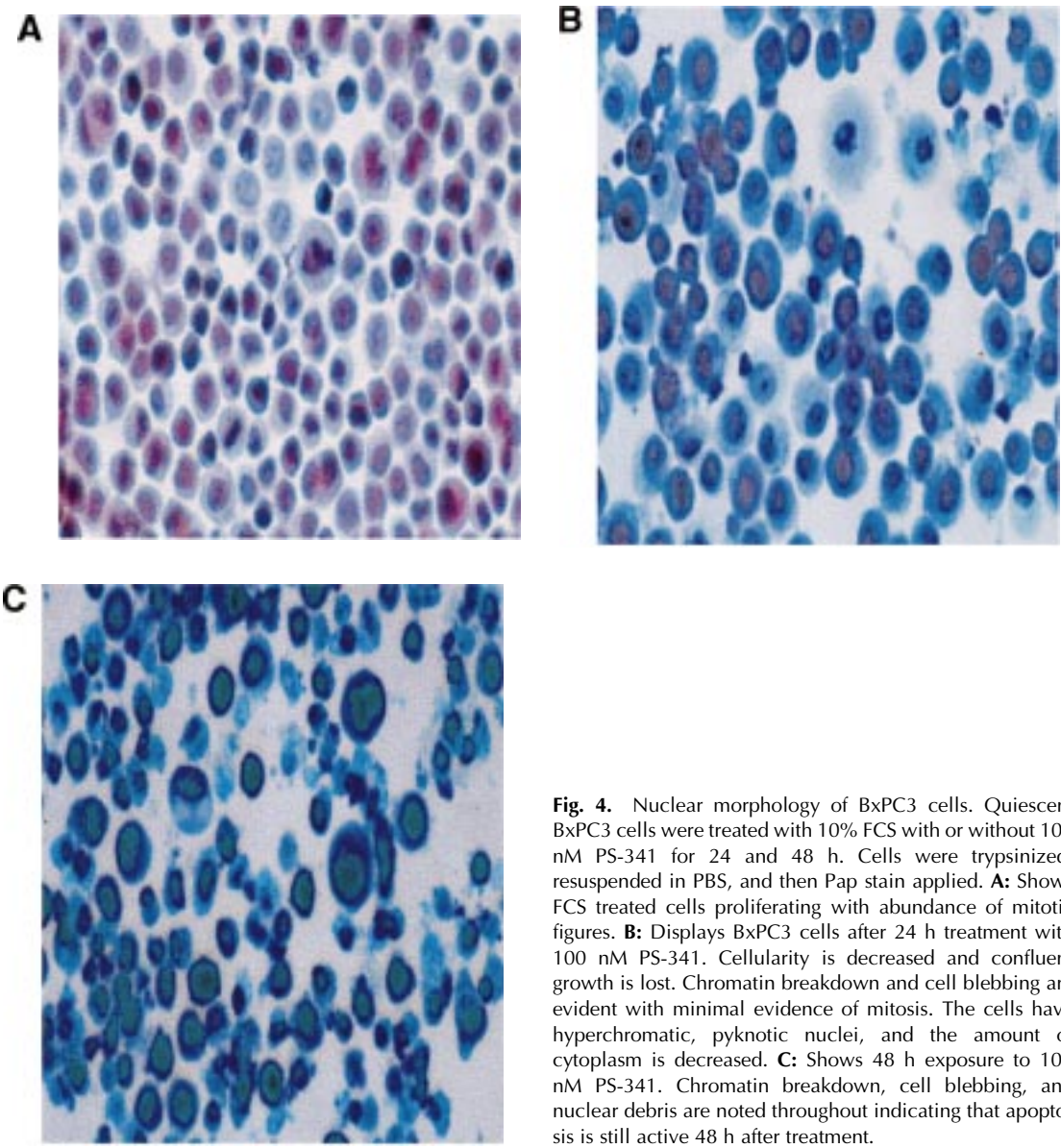

Fig. 4. Nuclear morphology of BxPC3 cells. Quiescent BxPC3 cells were treated with 10% FCS with or without 100 nM PS-341 for 24 and 48 h. Cells were trypsinized, resuspended in PBS, and then Pap stain applied. **A:** Shows FCS treated cells proliferating with abundance of mitotic figures. **B:** Displays BxPC3 cells after 24 h treatment with 100 nM PS-341. Cellularity is decreased and confluent growth is lost. Chromatin breakdown and cell blebbing are evident with minimal evidence of mitosis. The cells have hyperchromatic, pyknotic nuclei, and the amount of cytoplasm is decreased. **C:** Shows 48 h exposure to 100 nM PS-341. Chromatin breakdown, cell blebbing, and nuclear debris are noted throughout indicating that apoptosis is still active 48 h after treatment.

were treated weekly for four weeks with either 1 mg/kg IV or IP PS-341, or vehicle only, and tumor growth measured for 40 days. Systemic 26S proteasome inhibitor delivered either IP (84% reduction) or IV (72% reduction) significantly limited tumor growth ($^*P < 0.001$ vs. vehicle) as measured by relative tumor volumes (Fig. 6A). There was no difference in mortality or body weight among groups ($n = 12$/group), indicating that systemic PS-341 therapy was not toxic.

Tumor $p21^{Cip1\text{-}Waf\text{-}1}$ Levels

The cellular effect of systemic 26S proteasome inhibition was determined by immunoblotting of cytoplasmic protein extracts from tumor xenografts. Tumor levels of $p21^{Cip1\text{-}Waf\text{-}1}$, a cell cycle protein normally degraded by the 26S proteasome, were significantly greater in the PS-341 treated samples (Fig. 6B) indicating that this systemic therapy was effective at the cancer cell level.

26S Proteasome Inhibition Blocks CPT-11 Induced Degradation of IκB-α

CPT-11 is a topoisomerase I inhibitor which activates NF-κB [Wang et al., 1999; Cusack et al., 2000; Huang et al., 2000]. This agent is currently being tested in pancreatic cancer protocols, but with limited success [Kloep et al.,

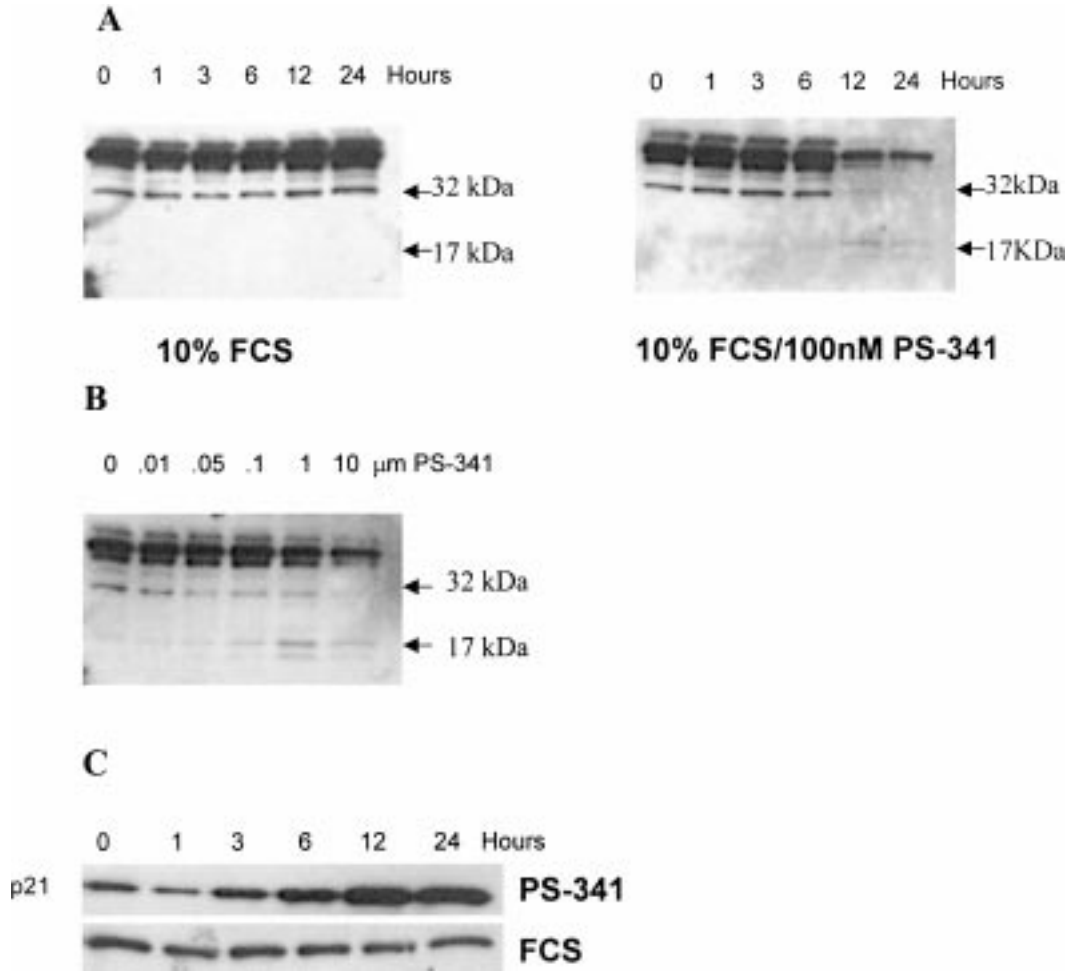

Fig. 5. Cell cycle checkpoint proteins. Following mitogen (10% FCS) stimulation with and without varying amounts of PS-341, cytoplasmic lysates were electrophoresed using 10–20% SDS-PAGE gels and caspase-3 activation (50 μg) and $p21^{Cip1\text{-}Waf\text{-}1}$ levels (12 μg) were determined by immunoblotting. Activation of caspase-3 is represented by cleavage of the inactive 32 kDa caspase-3 fragment to the active 17 kDa fragment. **A:** indicates caspase-3 activation in a 24 h time period comparing FCS with or without 100 nM PS-341. **B:** Indicates a dose response of PS-341 with 24 h treatment. **C:** Activation of $p21^{Cip1\text{-}Waf\text{-}1}$ by 3 h with 100 nM PS-341 represents activation of CDK inhibitors and cell cycle arrest.

1999]. The regulatory events governing the activation of NF-κB induced by cytokines involves cytoplasmic signaling cascades, namely the degradation of NF-κB inhibitor, IκB-α, by a ubiquitin-proteasome pathway. CPT-11 treatment, similar to TNF-α, caused a reduction in IκB-α protein levels (Fig. 7A and B) by 15 min, which was prevented by proteasome inhibition (Fig. 7D). PS-341 had not effect on IκB-α (Fig. 7C).

Enhancement of CPT-11 Tumoricidal Effect by 26S Proteasome Inhibition

To evaluate if systemic 26S proteasome inhibitor therapy would enhance pancreatic tumor sensitivity to CPT-11, we randomized (n = 10/group) athymic nu/nu mice with established (50 mm^3) subcutaneous BxPC3 human pancreatic cancer xenografts to four treatment groups. Mice received either (1) IV vehicle, (2) IV CPT-11 [33 μg/kg], (3) IP PS-341 [1 mg/kg], or (4) IV CPT-11, 33 μg/kg and IP PS-341, 1 mg/kg every four days for five treatments. After 40 days (Fig. 8A), systemic therapy with CPT-11 limited tumor growth 43% compared to vehicle. When combined with PS-341, this effect was significantly enhanced as pancreatic tumor volumes regressed by 89% compared to vehicle alone ($P < 0.001$, ANOVA). Tumors regressed completely in two of ten animals treated with CPT-11/PS-341, but slowly reappeared when the treatment ended. There was no difference in mortality or body weight among groups (n = 10/group), indicating that systemic combination

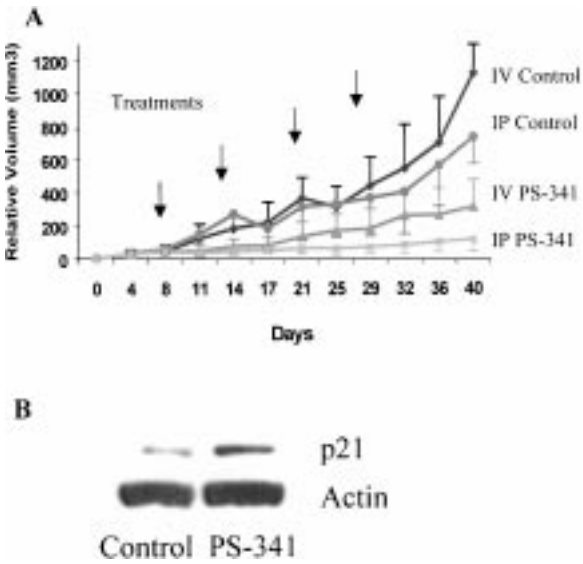

Fig. 6. Effect of systemic PS-341 on mouse BxPC3 tumor xenografts. **A:** Athymic nu/nu mice (n = 12/group) bearing established subcutaneous BxPC3 tumor xenografts (50 mm^3) were treated with four weekly injections of 1.0 mg/kg PS-341 (IV or IP) or vehicle (0.9% w/v sterile saline solution with 0.1% w/v ascorbic acid) as controls. Tumor volumes were measured along the longest orthogonal axes and calculated (see text). Relative tumor volume was calculated as tumor volume on the day measured divided by initial tumor volume (V_x/V_i). Significance was determined by ANOVA ($P < 0.001$, controls vs. PS-341). **B:** at the end of the tumor xenograft growth experiment (40 days), tumors were harvested, snap frozen in liquid nitrogen, and cytoplasmic extractions performed. 50 μg protein were electrophoresed using a 10–20% SDS-PAGE gel, and $p21^{Cip1\text{-}Waf\text{-}1}$ levels determined by immunoblotting. Membranes were stripped and rerun for actin to ensure equal protein loading and membrane transfer. This is a representative immunoblot of tumors harvested from three different animals from each group. No significant differences in $p21^{Cip1\text{-}Waf\text{-}1}$ expression were evident from IV to IP groups.

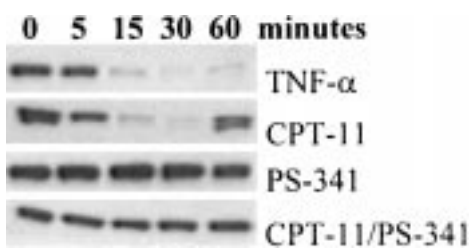

Fig. 7. 26S proteasome inhibition prevents IκB-α degradation induced by CPT-11 in vitro. Quiescent BxPC3 cells were treated with (A) TNF-α [400 U/ml], (B) CPT-11 [5 μM], (C) PS-341 [10 nM], or (D) combination CPT-11 [5 μM] and PS-341 [10 nM]. TNF-α and CPT-11 induced IκB-α degradation by 15 min. PS-341 stabilized IκB-α throughout the time course and prevented IκB-α degradation secondary to CPT-11 exposure.

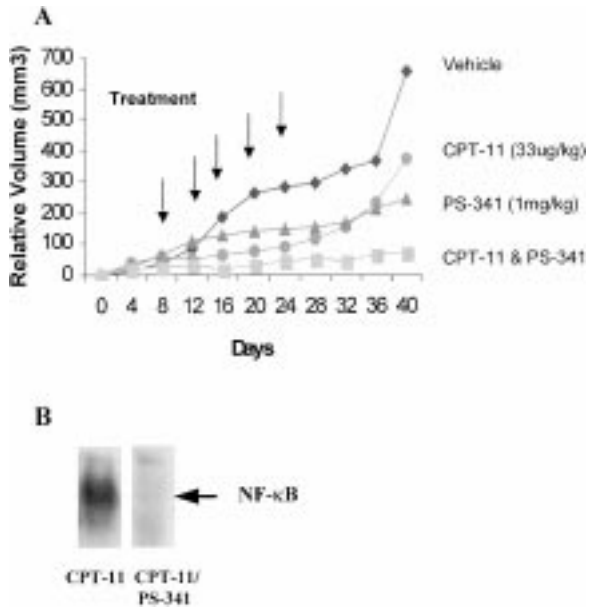

Fig. 8. Inhibition of tumor xenograft growth with CPT-11/PS-341 combination therapy. **A:** Established (50 mm^3) BxPC3 tumor xenografts in animals were treated with (1) vehicle, (2) IV CPT-11 alone [33 μg/kg], (3) IP PS-341 alone [1 mg/kg], or (4) combination therapy of IV CPT-11 [33 μg/kg] and IP PS-341 [1 mg/kg]. Treatment consisted of five injections every four days (arrows). Tumors volumes were measured along the longest orthogonal axes and calculated (see text). Relative tumor volume was calculated as tumor volume on the day measured divided by initial tumor volume (V_x/V_i). Significance was determined by ANOVA ($P < 0.001$). **B:** At the end of the tumor xenograft growth experiment (40 days), tumors were harvested, snap frozen in liquid nitrogen and nuclear extractions performed. 8 μg protein were loaded and EMSA run for NF-κB mobilization. This is a representative EMSA of tumors harvested from three different animals from each group.

CPT-11/PS-341 therapy was not toxic in the animals.

NF-κB Activation In Vivo

To investigate further the mechanism underlying the chemoresistance of pancreatic cancer xenografts to CPT-11 alone, nuclear protein of tumors was analyzed for NF-κB activation by EMSA (Fig. 8B). CPT-11 treatment alone, induced activation of NF-κB, while combination therapy of CPT-11/PS-341 blocked NF-κB activation. Vehicle and PS-341 treatment alone did not induce significant activation of NF-κB (not shown).

Induction of Apoptosis In Vivo

To determine if the enhanced anti-tumor effect realized with systemic proteasome inhibitor therapy resulted from tumor cell apopto-

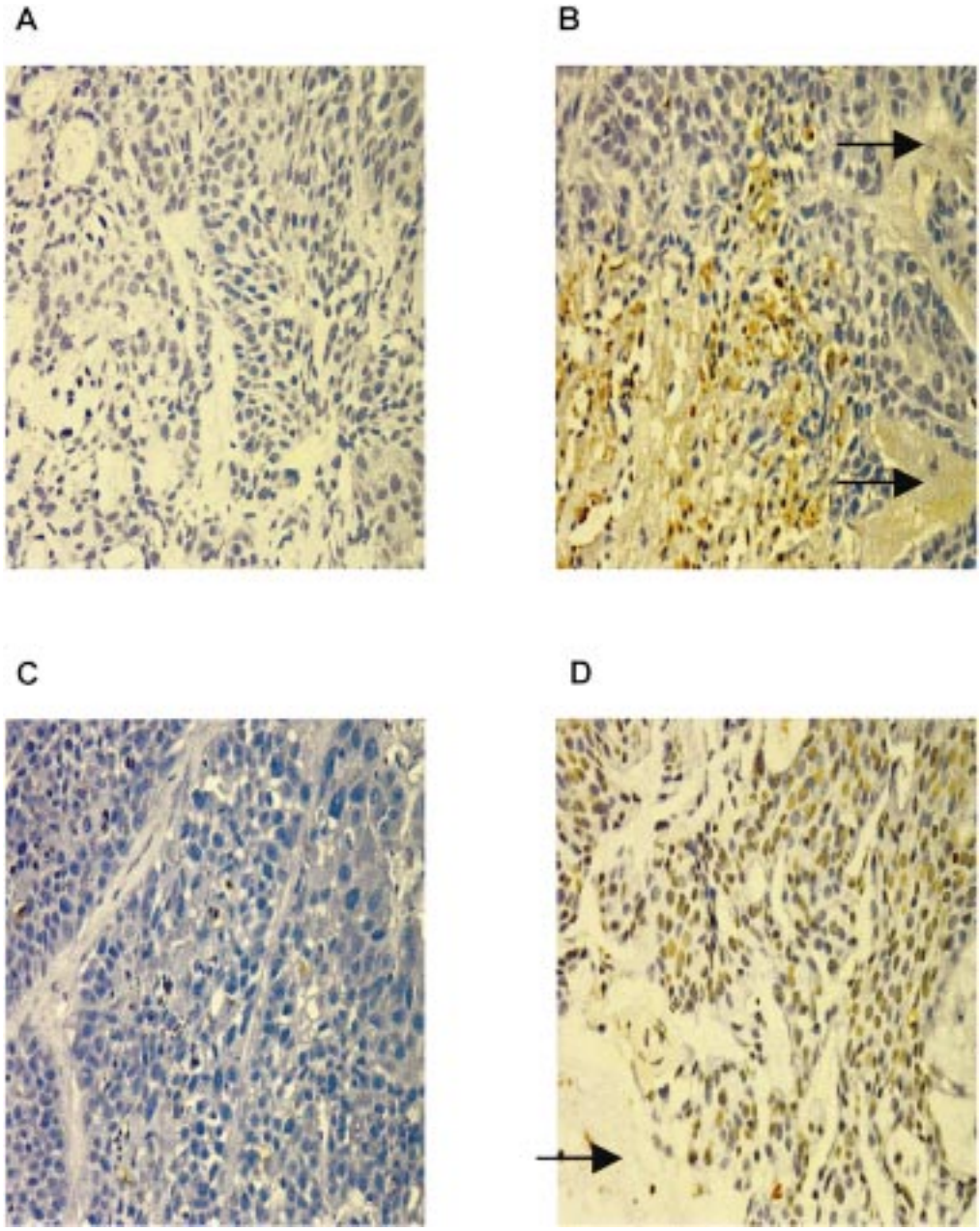

Fig. 9. In vivo Apoptosis in Tumor Xenografts. At the end of the two tumor xenograft growth experiments (40 days), tumors were harvested, fixed in 10% formalin, and then analyzed for apoptosis with TUNEL assay. Nuclei staining orange are considered apoptotic. Tumors treated with either vehicle **(A)** or CPT-11 **(C)** showed minimal evidence of apoptosis. In PS-341 treated tumors **(B)**, both apoptosis and necrosis (arrows) are increased compared with controls. Apoptosis is greatest in tumors controlled by CPT-11/PS-341 combination therapy **(D)**. Histology shown is representative of three different experiments.

sis, we harvested tumors from all groups at 40 days and processed them for histology and TUNEL assays. Tumors in animals treated with either IV or IP PS-341 (Fig. 9B) demonstrated more apoptosis and necrosis than did tumors in animals treated with vehicle alone (Fig. 9A). CPT-11/PS-341 combination therapy (Fig. 9D) yielded tumors with mostly apoptotic cells, while CPT-11 administration alone (Fig. 9C) did not.

26S Proteasome Inhibition Blocks Proliferation In Vivo

Tumor cell proliferation was assessed with fluorescent imaging of BrDU incorporation into proliferating tumor cells. Animals treated with

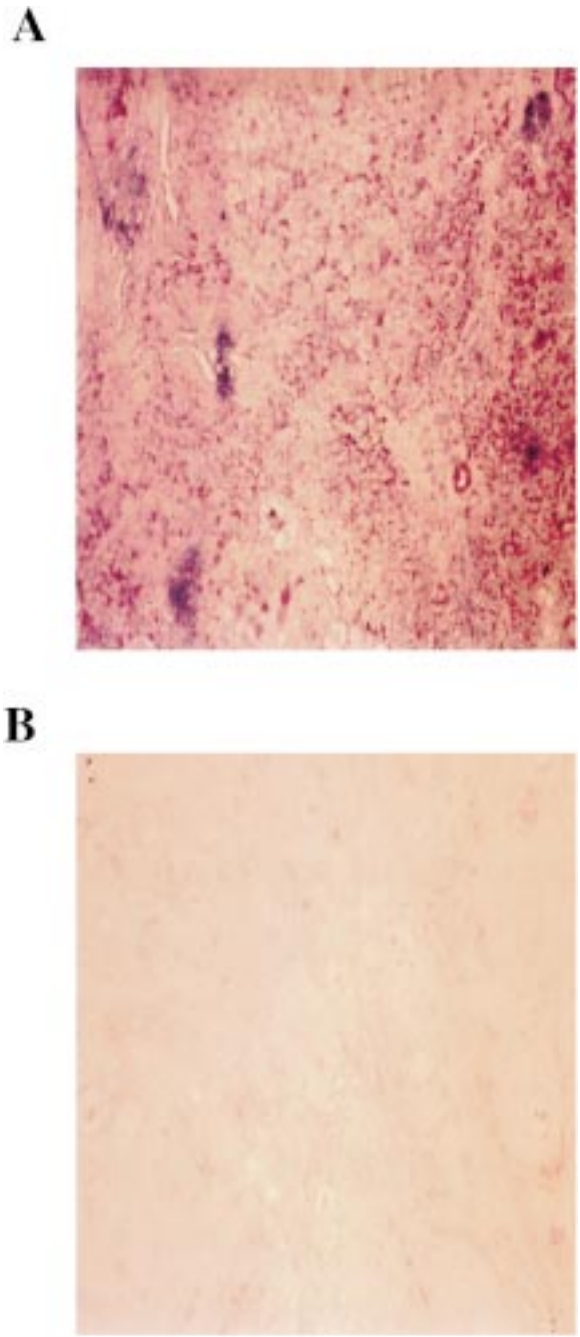

Fig. 10. In vivo proliferation in tumor xenografts. Nuclear BrDU incorporation followed by fluorescent staining was used to determine in vivo changes in tumor cell proliferation. At the end of the experiment, tumors from mice treated with either **(A)** IV CPT-11 alone [33 μg/kg] or **(B)** combination therapy of IV CPT-11 [33 μg/kg] and IP PS-341 [1 mg/kg] were harvested 1 h after intratumoral BrDU injection. With CPT-11 treatment alone (A), tumor cells stained dark red representing nuclear uptake of BrDU, while combination CPT-11/PS-341 treated cells had minimal BrDU incorporation. This represents inhibition of tumor cell proliferation in the combination therapy treated animals.

CPT-11 alone (Fig. 10A) stained dark red representing BrDU uptake into nuclei of cells, while combination CPT-11/PS-341 therapy prevented BrDU incorporation and stained pale white. In vivo BrDU correlates with in vitro proliferation data that not only does 26S proteasome inhibition block proliferation, but also enhances any anti-tumor effect of CPT-11 at the cellular level.

DISCUSSION

Targeted inhibition of the 26S proteasome blocked proliferation and induced apoptosis in BxPC3 pancreatic cancer cells and tumors. Pro-apoptotic kinases became activated. Systemic proteasome inhibition with PS-341 limited pancreatic tumor xenograft growth in nu/nu mice by 84% and significantly enhanced the tumoricidal effects of CPT-11. Apoptosis was consistently the hallmark of tumor cell death. Our study demonstrates for the first time the sensitivity of human pancreatic cancer to this novel biological therapy.

Patients and clinicians dealing with pancreatic cancer desperately need effective therapies. They face a stalemate recently highlighted by Sener et al. [1999] in a review of 1,00,313 patients. Specifically, overall survival rates have not changed in two decades. For the 10–15% of tumors actually resectable at the time of diagnosis, aggressive pancreatectomy confers at most a 23% five-year survival. In the other 85% of victims, five-year survival is only 5%, with the majority usually dead within months of diagnosis. The answers lie in the biology of the disease, and not whether it can be removed. Novel treatments such as gemcitabine [Berlin et al., 2000; Wiernik, 2000], human GM-CSF [Jaffee, 1999], p53 modulated gene therapy [Cascallo et al., 2000], and CPT-11 [Kloep et al., 1999] are only marginally effective against this lethal disease.

Apoptosis is a natural mechanism for cell death and plays a major role in regulating tumor growth. Enhancing apoptosis can lead to tumor regression. Ionizing radiation, certain chemotherapeutic agents, and tumor necrosis factor (TNF-α) can induce apoptosis, but their clinical efficacy is variable and side effects are significant [Klampfer et al., 1999]. These agents also simultaneously activate a NF-κB salvage pathway which limits apoptosis. Wang et al. [1996] reported that NF-κB activation by TNF-

α, radiation and daunorubicin inhibited apoptosis otherwise induced by these stimuli in fibrosarcoma cells. A cell survival response occurs through the induction of anti-apoptotic genes, such as IAP-1 and -2 [Wang et al., 1998]. Such resistance to apoptosis is a principle mechanism by which cancer cells escape death and grow unregulated [Fisher, 1994].

Like apoptosis, the ubiquitin-proteasome pathway is a highly regulated process [Grimm and Osbourne, 1999]. The 26S proteasome is an ATP dependent, multicatalytic protease that degrades oxidized or misfolded proteins as well as having other "housekeeping" activities. Specifically, the 26S proteasome degrades regulatory proteins that govern the cell cycle, transcription factor activation, apoptosis, and cell trafficking [King et al., 1996]. Altered degradation of cell cycle proteins can result in accelerated and uncontrolled cell division, promoting cancer growth and spread [Sherr, 1996].

The recent availability of proteasome inhibitors has demonstrated the need for proteasome activity during apoptosis in cycling and noncycling cells [Grimm and Osbourne, 1999] and in the regulation of cell viability and proliferation [Imajoh-Ohmi et al., 1995; Lopes et al., 1997; Teicher et al., 1999]. Inhibition of the 26S proteasome can lead to inhibition of cell growth through apoptosis. Proteasome inhibitors protected primary mouse thymocytes and sympathetic neurons from cell death caused by cytotoxic agents and nonviable cellular conditions [Grimm et al., 1996; Sadoul et al., 1996]. A collective pro-apoptotic effect of ubiquitin-proteasome inhibition appears to occur in proliferating but not quiescent cells, and may represent a unique biological strategy to treat human cancers.

Proteasome inhibitors are available in four classes: (1) lactacystin and β-lactone derivatives, (2) vinyl sulfones, (3) peptide aldehydes, and (4) peptide boronates. The aldehyde and boronate inhibitors are reversible and more amenable to clinical use [Adams and Stein, 1996; Lee and Goldberg, 1998; Adams et al., 1999; McDade et al., 1999]. PS-341, a dipeptide boronic acid analogue, specifically inhibits the chymotryptic activity of the 26S proteasome by blocking the enzyme [Adams et al., 1999]. Substantial work done by Adams et al. [1999] confirmed in vivo activity of PS-341 in nude mice and showed effective levels of 20S proteasome inhibition with once weekly dosing and shorter intervals. PS-341 and other proteasome inhibitors have been shown to inhibit proliferation in breast, prostate and lung cancers [Imajoh-Ohmi et al., 1995; Teicher et al., 1999]. We now demonstrate that 26S proteasome inhibition is tumoricidal in pancreatic cancer, a more refractory and chemoresistant tumor.

We determined the time course and kinetics for the effect of proteasome inhibition on BxPC3 cells. 26S proteasome inhibition blocked mitogen-induced proliferation, inhibited cell cycle entry, and induced apoptosis in pancreatic cancer cells in vitro. Morphologic examination of cells revealed that any changes in proliferation were due to apoptosis and not necrosis. Caspase-3 activation, an early indicator of apoptosis, was evident within 12 h of mitogen stimulation. Our proliferation assays involved a model of serum starvation to induce cell quiescence [Perugini et al., 2000]. Both MTT and BrDU assays showed that 26S proteasome inhibition blocked proliferation vs. FCS alone. 100 nM PS-341 appeared to be the optimal concentration to induce apoptosis for the time course given, without necrosis. Ten nanometer PS-341 had little if any effect on cellular proliferaiton and apoptosis when given alone, while 1,000 nM was cytotoxic. Finally, protein levels of $p21^{Cip1\text{-}Waf\text{-}1}$ revealed that a protein commonly degraded by the 26S proteasome was instead stabilized as early as 3 h with PS-341. This time period correlates with caspase-3 activation (12 h) indicating that growth arrest precedes apoptosis. The accumulation of $p21^{Cip1\text{-}Waf\text{-}1}$ may be an early event that denies the cell entry through the G_1-S transition. To confirm this finding, cell cycle analysis of proliferating pancreatic cancer cells with or without PS-341 was performed. After 12 h of exposure to PS-341 with 10% FCS as mitogen, cells were arrested in G_0-G_1. Although this differs from other studies with PS-341 [Adams et al., 1999], it supports the accumulation of $p21^{Cip1\text{-}Waf\text{-}1}$ inducing a G_0-G_1 cell cycle arrest.

Our animal tumor xenograft studies prove the antitumor effects of 26S proteasome inhibition in an in vivo model of human pancreatic cancer. At the completion of the study (40 days), we observed an 84% reduction in tumor growth with four weekly IP injections of proteasome inhibitor. In all groups, animals did not exhibit

any evidence of toxicity. To ensure that the reduction in tumor volume was due to apoptosis, we harvested the tumors and sectioned them longitudinally to include the center. Although we did not analyze serum levels of the 20S proteasome or drug levels [Adams et al., 1999], protein analysis of $p21^{Cip\text{-}Waf\text{-}1}$ was evaluated. Stabilization of this cyclin-dependent kinase inhibitor was considered not only to indicate cell cycle arrest, but also to confirm proteasome inhibition in tumor cells.

The topoisomerase I inhibitor CPT-11 and other DNA damaging agents have been shown to activate NF-κB in many cancer cell types [Wang et al., 1999; Cusack et al., 2000; Huang et al., 2000]. Inhibiting NF-κB activation by proteasome inhibition should potentiate the apoptotic response to CPT-11. Adenoviral delivery of a modified form of IκB-α was found to sensitize fibrosarcoma and colorectal tumor xenografts in nude mice to CPT-11 resulting in tumor regression and apoptosis. The authors concluded that activation of NF-κB is the principle mechanism of inducible tumor resistance [Wang et al., 1999; Cusack et al., 2000]. Proteasome inhibition in cultured CEM/C2 cells not only prevented IκB degradation, but also NF-κB activation in response to CPT-11 treatment [Huang et al., 2000].

We therefore investigated whether systemic 26S proteasome inhibition would enhance the sensitivity of pancreatic cancer xenografts to CPT-11. PS-341 prevented CPT-11 induced degradation of NF-κB inhibitor, IκB-α, preventing nuclear translocation of NF-κB. In vivo, we show that although CPT-11 alone limited tumor growth by 43%, combination therapy with PS-341 induced tumor regression by 89%. This therapy was not toxic to any of the animals. In this second xenograft study, PS-341 administered alone, limited tumor growth by 65%. Although less than measured in our first xenograft study (84%, Fig. 7), this anti-tumor effect was reproducible and profound. Nuclear protein analysis of harvested tumors revealed activation of NF-κB by CPT-11 alone, but this effect was blocked in animals treated with systemic combination therapy of CPT-11/PS-341. This implies that NF-κB may be responsible for pancreatic cancer xenograft chemoresistance to CPT-11 treatment. We are currently validating this effect in additional human pancreatic cancer cell lines and further post-translational studies.

In summary, we have shown that 26S proteasome inhibition induces apoptosis and inhibits growth of human pancreatic cancer. These experimental findings may provide rationale for evaluating PS-341, alone or combined with chemotherapy, in patients with pancreatic cancer.

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