Effects of the Proteasome Inhibitor PS-341 on Apoptosis and Angiogenesis in Orthotopic Human Pancreatic Tumor Xenografts

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Abstract
Recent studies have shown that the transcription factor, nuclear factor κB (NF-κB), regulates critical survival pathways in a variety of different cell types, including human pancreatic cancer cells. The activation of NF-κB is controlled by proteasome-mediated degradation of its endogenous polypeptide inhibitor, inhibitor of nuclear factor κBα. We investigated the effects of PS-341, a peptide boronate inhibitor of the proteasome in human pancreatic cancer cells in vitro and in vivo. Comparison of PS-341’s effects on the growth of eight different human pancreatic cancer cell lines revealed marked heterogeneity in drug responsiveness, ranging from highly resistant (IC_{50} > 10 μM; Panc-46, HS766T, and Mia-PaCa-2) to extremely sensitive (IC_{50} < 40 nM; L3.6pl, Hpafl2, and BxPC3). However, these effects did not correlate with differential inhibition of NF-κB activation. Direct quantification of apoptosis revealed that PS-341’s effects on cell growth largely correlated with sensitivity to programmed cell death. Evaluation of PS-341’s effects on established orthotopic tumor xenografts demonstrated that biweekly intravenous administration of the maximum-tolerated dose of the drug (1 mg/kg) led to significant reductions in the volumes of L3.6pl tumors but not Mia-PaCa-2 tumors. Laser scanning cytometer-mediated quantification of drug-induced apoptosis in the xenografts confirmed that PS-341 induced DNA fragmentation and activation of caspase-3 in L3.6pl tumors but not in Mia-PaCa-2 tumors. However, histological examination of drug-treated tumors revealed extensive central necrosis and reductions in microvessel density and VEGF expression in both tumor types. Taken together, our results demonstrate that PS-341 inhibits the growth of human pancreatic tumors via direct effects on tumor cells and indirect effects on the tumor vasculature.

Introduction
Pancreatic carcinoma is the fourth most common cause of cancer-related death among males and the fifth most common among females in the United States (1). Prognosis remains very poor, with 5-year survival rates of <5% (2). Challenges to effective treatment of the disease include a lack of early screening strategies and the ineffectiveness of current therapeutic regimens. The vast majority of patients present with metastatic disease, and even where surgical resection of localized disease is possible, adjuvant chemotherapy and radiation therapy is not very effective, extending survival by an average of 3–6 months (3). New, more effective treatment strategies are clearly needed.

Recent investigation of the molecular events associated with pancreatic cancer progression has identified a number of genetic abnormalities that could represent outstanding targets for therapeutic intervention (4). Mutational activation of the K-ras oncogene is among the most prevalent, occurring in up to 90% of tumors (5, 6). The pleiotropic effects of K-ras activation may include activation of the transcription factor, NF-κB,3 a well-known target of Ras activation in other model systems (7, 8). Indeed, preliminary results demonstrate that NF-κB is constitutively activated in a majority (>70%) of human pancreatic cancer cell lines and primary tumor specimens (9). Activation of NF-κB has been implicated in suppression of cytokine- and chemotherapy-induced apoptosis (10–12), and it is therefore an excellent candidate modulator of drug resistance in the disease.

The ubiquitin-proteasome pathway is responsible for most intracellular protein degradation (13). Specificity is accomplished by conjugation of protein substrates with polymers of the M, 8,000 polypeptide, ubiquitin (14). Although many proteasome substrates have been identified, one of the most important is IκBα (15, 16). IκBα is an inhibitor of NF-κB that binds to the transcription factor’s nuclear localization domain, preventing it from translocating...

3 The abbreviations used are: NF-κB, nuclear factor κB; IκB, inhibitor of nuclear factor κBα; NCgl, National Cancer Institute; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AMC, 7-amino-4-methylcoumarin; FACS, fluorescence-activated cell sorter; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor.
ing to the nucleus and promoting expression of NF-κB’s target genes (17). Ubiquitin conjugation is stimulated by phosphorylation of IκBα on two conserved serine residues (18, 19). Proteasome inhibitors block IκBα degradation and subsequent NF-κB activation (15, 16). Importantly, proteasome inhibitors also stimulate apoptosis in a variety of tumor cell types (20, 21; reviewed in Ref. 22). Given the established role of NF-κB in suppression of apoptosis, it is likely that NF-κB inhibition contributes to proteasome inhibitor-induced cell death.

PS-341 is a dipeptide boronate antagonist of the proteasome that was recently developed by ProScript, Inc. (now Millennium Pharmaceuticals), for use in cancer therapy. PS-341 forms a covalent, reversible complex with the proteasome and is much more potent than its peptide aldehyde predecessors (i.e., MG-132 and calpain inhibitor-I; Ref. 23). Preclinical analysis of PS-341’s growth inhibitory activity against NCI’s panel of 60 tumor cell lines demonstrated an average GI₅₀ of 7 nM, placing the compound among the top 10 agents with respect to potency tested to date in the screen (23). In vivo drug distribution studies demonstrated that PS-341 accumulates in several gastrointestinal tissues, including the pancreas (23). This fact, combined with the recent implication of NF-κB activation in pancreatic cancer, prompted us to study the effects of PS-341 on established orthotopic human pancreatic tumors in nude mice.

Materials and Methods

Animals, Cell Lines, and Antibodies. Male nude mice (BALB/c background) were purchased from the Animal Production Area of the NCI-Frederick Cancer Research and Development Center (Frederick, MD). The human pancreatic cancer cell lines MiaPaCa-2, Aspc1, Hs766t, L3.6pl and Panc-48 human pancreatic adenocarcinoma lines were derived from COLO-357 and Panc-1, respectively, following selection for metastasis from the pancreas to the liver in nude mice (24, 25). Cell lines were maintained in MEM supplemented with 10% FBS, antibiotics, vitamins, and pyruvate under an atmosphere of 5% CO₂ in air. Antibodies were obtained from the following commercial sources: anti-IκBα and phospho-IκBα (S32; Cell Signaling, Inc., Beverly, MA); rat antimonouse CD31/PECAM-1 (Parr-Mingen, San Diego, CA); mouse antihuman proproliferating cell nuclear antigen (clone PC 10; Dako A/S, Glostrup, Denmark); antimonouse IgG (Amersham, Arlington Heights, IL); peroxidase conjugated F(ab)² goat antirabbit IgG F(ab)² and peroxidase-conjugated goat antirat IgG (H+L) (Jackson Research Laboratories, West Grove, CA); and peroxidase conjugated rat antimonouse IgG2a (Sero tec; Harlan Bioproducts for Science, Inc., Indianapolis, IN).

Viability Analysis. Cells were harvested in their exponential growth phase by a 2-min treatment with 0.25% trypsin/0.02% EDTA (w/v). (When this procedure was followed, the viability of harvested cells always exceeded 95%.) Cells were seeded into 96-well microculture plates at 5000 cells/well and allowed to attach for 24 h. The medium was removed and replaced with normal medium with or without increasing concentrations of PS-341, and cells were incubated for 48 h. The medium was then removed and replaced with fresh medium without the cytotoxic agents, and cells were incubated for an additional 24 h. Cell survival/cytostasis was then quantified using the tetrazolium dye MTT as described previously (26). Each experimental data point represented average values obtained from eight replicates, and each experiment was performed in triplicate. Viability measurements were confirmed by trypan blue exclusion.

Quantification of DNA Fragmentation. DNA fragmentation was measured by propidium iodide staining and FACS analysis as described previously (27). After incubation in vitro, cells were harvested, pelleted by centrifugation, and resuspended in PBS containing 50 µg/ml propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate. Samples were stored at 4°C for 16 h and vortexed before FACS analysis (FL-3 channel; Becton-Dickenson FACScan, Mountain View, CA).

Caspase Assay. Cells were plated in 6-well plates with MEM with 10% FBS and were allowed to attach for 24 h. Cells were treated with 10 µM PS-341 or 1 µM staurosporine for 24 h. After treatment, cells were lysed with 200 µl of caspase lysis buffer [100 µM HEPES (pH 7.4), 1% sucrose, 0.1% Chaps, and 1 mM EDTA] with a Complete Mini protease inhibitor tablet (Boehringer, Indianapolis, IN). Lysate was added to 800 µl of caspase buffer and 2 µl of 25 mM AMC fluorogenic substrate (Enzyme Systems Products, Livermore, CA) and incubated for 1 h at 37°C. After incubation, 1 ml of caspase buffer was added to dilute the mix and the samples were read on a Shimadzu spectrofluorophotometer (Model RF-1501).

20S Proteasome Activity Assay. The effects of PS-341 on proteasome activity were measured as described previously (28). Briefly, cell pellets were suspended in ice-cold 5 mM EDTA (pH 8.0) and lysed for 15 min on ice. Debris was sedimented by centrifugation at 16,000 × g for 5 min. Protein contents were determined using the Bradford assay. Lysate volumes equivalent to 10–50 µg of protein were mixed with an assay buffer containing 80 µM Suc-Leu, Leu, Val, Tyr (LLVY)-AMC in 20 mM HEPES (pH 8.0), 0.5 mM EDTA, 0.035% SDS, and 1% DMSO. Reaction progress was monitored by the increase in fluorescence emission at 440 nm (380 nm excitation). Data were collected for 5 min/sample.

Quantification of VEGF Secretion by ELISA. To evaluate VEGF expression after treatment with PS-341, cells were plated in 6 well plates with 2 ml of MEM with 1% FBS. Twenty-four h later, cells were exposed to 100 nM, 500 nM, or 1 µM of PS-341 for 24 h. Supernatants were collected, and VEGF protein levels were determined using Quantikine ELISA kits (R&D Systems, Inc., Minneapolis, MN). Cell numbers were equivalent in control and PS-341-treated samples.

Immunoblotting. Cells were collected at ~75–80% confluence with trypsin and counted in triplicate. The cells were then lysed as described previously (29). Approximately 20 µg of total cellular protein from each sample was subjected to SDS-PAGE, proteins were transferred to nitrocellulose membranes, and the membranes were blocked with 5% nonfat milk in a
Tris-buffered saline solution containing 0.1% Tween 20 for 2 h at 4°C. The blots were then probed overnight with relevant antibodies, washed, and probed with species-specific secondary antibodies coupled to horseradish peroxidase. Immunoreactive material was detected by enhanced chemiluminescence (West Pico, Pierce, Inc., Rockville, IL).

**Orthotopic Injections.** Intrapancreatic human tumors were established as described previously (24). Cells were harvested from culture flasks after brief trypsinization and transferred to serum-free HBSS. Only single-cell suspensions of >90% viability determined by trypan blue exclusion were used. Male nude mice were anesthetized with methoxyurethane, a small left abdominal flank incision was made, and the spleen was exteriorized, and tumor cells (1 x 10^6 cells) were injected into the subcapsular region of the pancreas using a 30-gauge needle and a calibrated push button-controlled dispensing device (Hamilton Syringe Company, Reno, NV). A successful subcapsular intrapancreatic injection of tumor cells was controlled by the appearance of a fluid bubble without i.p. leakage. To prevent leakage, a cotton swab was held cautiously for 1 min over the site of injection. The abdominal wound was closed in one layer with wound clips (Autoclip; Clay Adams, Parsippany, NJ).

**Treatment Schedule.** Tumors were established for 7–14 days. Animals were then injected with PS-341 (100 µL, in saline, i.v. or i.p.) at doses of 0.3, 0.6, 0.8, or 1.0 mg/kg twice weekly for a total of four to six injections. Previous studies conducted by Millennium (detailed in their PS-341 Investigators’ Brochure) and our own experience indicated that the effects of i.p. versus i.v. drug administration on tumor growth and toxicity were very similar. The i.p. route was added to this study because of the technical difficulty encountered when performing multiple tail vein injections. Mice were killed by cervical dislocation. Primary tumors in the pancreas were excised and weighed. Lymph nodes and livers were also harvested for analysis of metastasis. For immunohistochemistry and H&E staining procedures, one part of the tumor tissue was formalin-fixed and paraffin-embedded, another part was harvested from culture flasks after brief trypsinization and fixed in xylene, followed by treatment with a graded series of alcohol [100%, 95%, and 80% ethanol/ddH_2O (v/v)] and rehydrated in PBS (pH 7.5), treated with pepsin (Biomeda) for 15 min at 37°C, and washed with PBS. Immunohistochemical procedures were performed as described previously (31). Positive reactions were visualized by incubating the slides with stable 3,3'-diaminobenzidine for 10–20 min. The sections were rinsed with distilled water, counterstained with Gill’s hematoxylin (colorimetric development), and mounted with Universal Mount (Research Genetics). Control samples exposed to secondary antibody alone showed no specific staining. Staining intensity was quantified by densitometric analysis of five random high-power fields containing viable tumor cells, and results correspond to the average absorbance.

**Results**

**PS-341 Induces Growth Arrest in Human Pancreatic Adenocarcinoma Cells.** Previous work showed that PS-341 induces growth arrest (IC_{50} = 20 nm after 48-h exposure) in the human prostate cancer cell line PC-3 *in vitro* (23). In the NCI panel of 60 cell lines, the mean IC_{50} of PS-341 was found to be 7 nm (23). Included in this panel are lines derived from a wide variety of human solid tumors, but human pancreatic cancer was not represented (32). To determine whether human pancreatic cancer cells are also sensitive to PS-341, we exposed a diverse panel of cell lines (n = 8) to increasing concentrations of PS-341 for 48 h, and growth inhibition/cytotoxicity was measured 24 h later by MTT analysis. Somewhat unexpectedly, the cell lines displayed marked heterogeneity in drug responsiveness. Some of the cell lines (L3.6pl, HpaF2, and BxPC3) were markedly sensitive to the drug (IC_{50} < 40 nm), whereas others (Panc-48, HS766T, and Mia-PaCa-2) were extremely resistant (IC_{50} > 10 μM, Fig. 1A). The effects of PS-341 were associated with modest accumulation of cells at the G2-M phase of the cell cycle and increased expression of the cell cycle inhibitors p21 and p27 (data not shown), indicating that the drug inhibited cell cycle progression.

Several recent studies have shown that proteasome inhibitors induce apoptosis in other tumor cell types (20, 21, 23, 33). We therefore measured PS-341-induced DNA fragmentation at 48 h in our panel of pancreatic cancer cell lines (Fig. 1B). In general, the results of these analyses closely paralleled the results of the MTT analyses, indicating that the PS-341-induced growth inhibition was largely because of cell death. The cell line that displayed divergent responses was Aspc1; in these cells, PS-341 produced strong inhibition of growth (IC_{50} = 50 nm) but little to no apoptosis (compare Fig. 1, A and B). Additional evidence for drug-induced ap-
optosis was obtained from measurements of caspase-like protease activity (hydrolysis of Asp, Glu, Val, Asp (DEVD)-AMC), which was strongly induced in the L3.6pl cells but not in the Mia-PaCa-2 or AsPC-1 cells (Fig. 1C). Importantly, PS-341 was equally effective in blocking 20S proteasome activity in the L3.6pl and Mia PaCa-2 cells (Fig. 1D), indicating that the resistance of the Mia PaCa-2 cells to drug-induced apoptosis was not because of differences in drug targeting.

PS-341 Blocks Activation of NF-κB. Recent studies have shown that NF-κB is constitutively activated in human pancreatic cell lines and tumors, perhaps because of K-ras activation (9). Proteasome inhibition can reverse NF-κB activation via stabilization of the NF-κB inhibitor IκBα (15, 16). Consistent with these observations, incubation with PS-341 led to time-dependent accumulation of a serine phosphorylated form of IκBα in the L3.6pl and Mia PaCa-2 cells (Fig. 2A). Furthermore, PS-341 inhibited constitutive and TNF-induced NF-κB DNA binding activity in L3.6pl cells (Fig. 2B). Finally, PS-341 reduced both basal and TNF-induced NF-κB transcriptional activity, measured using a synthetic (3×NF-κB response element) promoter-reporter construct (Fig. 2C). Taken together, these results confirm that PS-341 inhibited NF-κB in both cell lines. The data also demonstrate that the drug blocks NF-κB activity to an equivalent extent in drug-sensitive (L3.6pl) and drug-resistant (Mia PaCa-2) cells. Therefore, the differences in PS-341-induced apoptosis observed in these cells are not because of differences in the drug’s effects on NF-κB.

Effects of PS-341 on Orthotopic Tumors. We next analyzed PS-341’s activity and toxicity in orthotopic models of metastatic human pancreatic cancer in nude mice. The drug was delivered biweekly via i.v. (tail vein) or i.p. injection because previous work has shown that 20S proteasome activity recovers to control levels by 72 h (23). Preliminary studies indicated that PS-341 toxicity in control and tumor-bearing mice treated
with doses of the drug higher than 1.0 mg/kg involved a >25% decrease in total body weight, diarrhea, and histopathological signs of gastrointestinal inflammation; doses > 1.5 mg/kg were not tolerated and led to rapid lethality. We therefore selected 1 mg/kg as the maximum-tolerated dose for subsequent studies. This maximum-tolerated dose is consistent with earlier reports (23, 34). Animals treated at this dose level displayed minimal weight loss (Table 1) or other signs of toxicity (data not shown).

In the first experiment we determined the effects of chronic PS-341 treatment on tumorigenicity and metastasis. Established (7-day) L3.6pl or Mia-PaCa-2 tumors were treated with 1.0 mg/kg twice weekly for a total of six injections. Animals were sacrificed on day 23, and primary tumors and metastases were analyzed. Divergent effects of the drug were noted in the two tumor models. In L3.6pl tumors, PS-341 did not affect tumor incidence or rates of metastasis to the lymph nodes or liver, but it did significantly inhibit tumor growth (Table 1). In contrast, PS-341 did not significantly affect the mean weights of the Mia-PaCa-2 tumors (Table 1). (Spontaneous lymph node or liver metastases were never observed in mice bearing Mia PaCa-2 tumors.)

The divergent effects of PS-341 on L3.6pl and Mia-PaCa-2 tumors closely correlated with the patterns of drug sensitivity established in vitro and suggested that differential induction of apoptosis might be involved. To directly test this possibility, mice bearing established (14-day) tumors were treated with one dose of PS-341 (1 mg/kg), and levels of apoptosis were measured by TUNEL (Fig. 3, A and B) or with an antibody to the active form of caspase-3, a cysteine protease that is important for the effector phase of apoptosis (Fig. 3, C and D). PS-341 induced significant increases in DNA fragmentation and caspase activation in the L3.6pl tumors (P < 0.05) but not in the Mia-PaCa-2 tumors (Fig. 3). Thus, the effects of PS-341 on tumor growth correlate well with levels of drug-induced tumor cell apoptosis.

Although PS-341 did not significantly inhibit the growth of Mia-PaCa-2 tumors, gross examination of tissue histology by...
PS-341 Inhibits Tumor Angiogenesis

Table 1  Effects of PS-341 on established orthotopic pancreatic tumors

<table>
<thead>
<tr>
<th>Tumor incidence</th>
<th>LN metastasis</th>
<th>Liver metastasis</th>
<th>Pancreas weight (g)</th>
<th>Tumor volume (mm³)</th>
<th>Body weight (g)</th>
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<tr>
<td>L3.6pl, control</td>
<td>9 of 8</td>
<td>7 of 8</td>
<td>3 of 8</td>
<td>1.5 (1.2–2)</td>
<td>432.7 (219–1331)</td>
</tr>
<tr>
<td>L3.6pl, PS341</td>
<td>7 of 7</td>
<td>7 of 7</td>
<td>1 of 7</td>
<td>0.74 (0.6–1.6)</td>
<td>168.7 (100–373)</td>
</tr>
<tr>
<td>MiaPaCa2, control</td>
<td>10 of 10</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1.07 (0.76–1.5)</td>
<td>327.4 (150–756)</td>
</tr>
<tr>
<td>MiaPaCa2, PS341</td>
<td>10 of 10</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.9 (0.45–1.8)</td>
<td>306.2 (125–800)</td>
</tr>
</tbody>
</table>

* Orthotopic tumors were generated by injecting 1 x 10⁶ cells into the pancreas of nude mice. Tumors were established for 7 days before the initiation of therapy with PS-341 (1 mg/kg twice weekly via i.v. injection). Animals were sacrificed 21 days later for assessment of tumor burden and analysis of biological endpoints. N.D., not detectable by gross histological analysis. Data ranges are indicated in parentheses.

Discussion

Accumulating evidence indicates that a series of discrete molecular alterations underlies the progression of human pancreatic cancer (35), and recent work has established that the transcription factor, NF-κB, is constitutively activated in a majority (>70%) of pancreatic cancer cell lines and primary patient tumors (9). Although the mechanism(s) leading to NF-κB activation have not been identified, the transcription factor is an attractive therapeutic target for several reasons. NF-κB regulates the expression of a wide variety of genes implicated in proliferation, angiogenesis, invasion, and metastasis (36, 37), and interruption of this signaling would be expected to inhibit all of these processes. Furthermore, many studies have shown that NF-κB inhibits apoptosis (10), and several of the genes involved in this death inhibitory activity have been identified (11). Finally, many conventional cancer therapies activate NF-κB as a byproduct of their effects on cancer cells, and this effect can limit their efficacy (12). These observations prompted us to search for small molecules that might interfere with the constitutive NF-κB activation observed in pancreatic cancer. The proteasome inhibitor, PS-341, represented the first such compound because proteasome-mediated degradation of NF-κB’s physiological inhibitors (the IκB proteins) is a well-known component of NF-κB activation. PS-341 is also attractive because it has already been evaluated in Phase I clinical trials in other solid malignancies (i.e., prostate cancer), where it displayed promising activity and minimal toxicity.⁴

We first evaluated PS-341’s effects in a panel of eight pancreatic cancer cell lines commonly used in our laboratory and others. PS-341 inhibits the isolated 20S proteasome with a Kᵦ of 0.6 nm, and it inhibits 20S proteasome activity in whole cells with a Kᵦ of ~7 nm (23). Given the strong activity observed in the NCI’s screen, we expected that PS-341 would induce apoptosis in all of our cell lines at low concentrations. Surprisingly, our results revealed marked heterogeneity in cellular responses: some cell lines displayed the predicted sensitivity to low concentrations of PS-341, whereas an equal number was refractory to very high concentrations of the drug. We did not conduct clonogenic assays to determine the long-term effects of PS-341 on cell viability; given that complete proteasome inhibition is incompatible with cell viability in organisms as divergent as bacteria and yeast and mammalian cells, all of our cell lines probably would have ultimately died in the presence of high concentrations of the drug. Nonetheless, given that complete 20S inhibition cannot be achieved without mortality in vivo (23), our data strongly suggest that some pancreatic tumor cells will display in-
herent resistance to the drug under the conditions that can be achieved in vivo. Direct measurement of 20S proteasome activity confirmed that PS-341 produced equivalent proteasome inhibition in drug-sensitive and drug-resistant cells (Fig. 1D), indicating that drug resistance was not because of differences in drug uptake or proteasome subunit composition. PS-341 also inhibited NF-κB in both drug-sensitive and -resistant cells (Fig. 2), providing additional evidence that it interacted with its biological target. Therefore, it appears that PS-341-resistant cells are better able to tolerate 20S proteasome inhibition than drug-sensitive cells. Importantly, drug resistance does not appear to be because of differences in NF-κB’s activation status before or after therapy.

We next measured PS-341-induced DNA fragmentation in our panel of cell lines to obtain quantitative information about its effects on apoptosis. Not unexpectedly, we observed a fairly close correlation between the drug’s effects on cell growth and its ability to induce apoptosis in that cell lines that were scored sensitive in the MTT assays also displayed high levels of PS-341-induced DNA fragmentation. The only cell line that deviated from this pattern was Aspc-1, which was strongly growth inhibited but did not undergo apoptosis. We considered the possibility that PS-341 killed the Aspc-1 cells by necrosis (without DNA fragmentation) but found no evidence for drug-induced increases in plasma membrane permeability (measured by

![Graphs and images](image-url)
Because Aspc-1 cells proliferate rapidly, we suspect that PS-341 acts as a pure cell cycle inhibitor in the cell line, probably via stabilization of p21, p27, and other cell cycle-active proteins. Determining whether or not this activity is sufficient to inhibit tumor growth in vivo will require additional experiments with tumor xenografts.

Our results confirmed earlier reports showing that proteasome inhibitors, and PS-341 in particular, block chemotherapy- or cytokine-induced NF-κB activation in cancer cells (34). However, the observation that PS-341 inhibited NF-κB equally well in drug-sensitive and -resistant cells (Fig. 2) demonstrates that inhibition of NF-κB per se is not always sufficient to induce apoptosis. On the other hand, it is possible that NF-κB inhibition lowers the threshold for apoptosis induced by other stimuli, including conventional cancer chemotherapeutic agents. Consistent with this idea, recent work by Cusack et al. (34) has demonstrated that PS-341 sensitizes colon cancer cells to CPT-11, and PS-341 + CPT-11 combinations produce better than additive effects on the growth of Lovo colon tumor xenografts compared with therapy with either drug alone. In our own hands, PS-341 appears to synergize with gemcitabine to inhibit the growth of human 253J-BV bladder cancer xenografts.\(^5\) In ongoing work, we are characterizing the effects of PS-341-based combinations on apoptosis in vitro and on tumor growth in vivo.


Fig. 4. Effects of PS-341 on tumor histology. Representative tumor sections obtained from tumors harvested from mice treated with the indicated doses of PS-341 after staining with H&E. A, L3.6pl tumors. B, Mia PaCa-2 tumors. C, quantification of necrotic areas by densitometry. Mean ± SD, \(n = 5\).
vivo in our pancreatic cancer cells and orthotopic tumor models.

Analysis of PS-341’s distribution in whole rats demonstrated that the drug accumulates in gastrointestinal tissues, including the pancreas (23). This fact coupled with our in vitro findings prompted us to evaluate PS-341’s activity in two different orthotopic models. The models were selected based on their widely divergent responses in vitro: the metastatic L3.6pl line was among the most drug sensitive, whereas the more well-differentiated Mia-PaCa-2 line was refractory. The outcome of our experiments was not obvious because our in vitro studies did not evaluate possible effects of PS-341 on host tissues, and other work has shown that cells growing in three dimensions (i.e., spheroids) respond differently to chemotherapy than do cells growing in monolayers (38). Nonetheless, the in vitro differences in drug sensitivity were largely recapitulated in the responses of the tumor xenografts to PS-341 therapy in vivo, suggesting that the intrinsic drug resistance identified in some of the cell lines did present a barrier to direct PS-341-induced cell killing in the Mia-PaCa-2 tumors. Importantly, however, histological examination of the Mia-PaCa-2 tumors revealed extensive central necrosis associated with immunohistochemical evidence of angiogenesis inhibition. As noted earlier, the expression of several proangiogenic factors (VEGF, IL-8, and MMP-9) is regulated by NF-κB, and PS-341 inhibited production of VEGF by Mia-PaCa-2 and L3.6pl cells in vitro. Our results are consistent with data obtained by another group who recently showed that PS-341 inhibited angiogenesis in human squamous cell carcinomas, effects that were also linked to inhibition of NF-κB (39). PS-341’s antiangiogenic

![Fig. 5. Effects of PS-341 on tumor angiogenesis. Sections from control or PS-341-treated tumors were stained with an antibody to CD31 to visualize the tumor vasculature. A, L3.6pl tumors. B, Mia PaCa-2 tumors. C, quantification of tumor microvessel density. Mean ± SD, n = 5.](image-url)
effects may therefore be of value even in tumors that are refractory to the direct cytotoxic effects of the drug. As is true with other biologically active drugs, it is likely that PS-341’s antiangiogenic effects will be best exploited within the context of combination chemotherapy.

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McConkey, D. J., Evans, D. B., Abbruzese, J. L., Hicklin, D. J., and
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Correction: Effects of the proteasome inhibitor PS-341 on apoptosis and angiogenesis in orthotopic human pancreatic tumor xenografts

In this article (Mol Cancer Ther 2002 1(14):1243-53), published in the December 1, 2002 issue of Molecular Cancer Therapeutics (1), the same images were used to represent multiple treatment conditions in Fig. 4A and B:

(1) The same image was used to represent treatment of L3.6pl (Fig. 4A) and Mia PaCa-2 (Fig. 4B) with 0.6 mg/kg PS-341 as well as treatment of Mia PaCa-2 with
The same image was used to represent treatment of L3.6pl (Fig. 4A) and Mia PaCa-2 (Fig. 4B) with 1.0 mg/kg PS-341.

The same image was used to represent drug control in L3.6pl (Fig. 4A) and Mia PaCa-2 (Fig. 4B).

The authors have corrected the figures and regret their errors. The corrected version is displayed on the previous page.

Corrections for several other articles published in AACR journals containing similar errors have been published. The original articles and their corrections are:


The errors in the aforementioned articles were caused in large part by inadequate oversight of data management. In an effort to prevent such errors from occurring in the future, the PI (DJM) has implemented a new data management policy that is designed to limit or eliminate errors in future publications and can be provided upon request (dmcconke@mdanderson.org). The authors note that the conclusions and interpretation of the data in these articles are unaltered by the errors that are now corrected.

Reference

Effects of the Proteasome Inhibitor PS-341 on Apoptosis and Angiogenesis in Orthotopic Human Pancreatic Tumor Xenografts


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