Proteasome Inhibitor PS-341 Inhibits Human Myeloma Cell Growth in Vivo and Prolongs Survival in a Murine Model


ABSTRACT

The proteasome is a ubiquitous and essential intracellular enzyme that degrades many proteins regulating cell cycle, apoptosis, transcription, cell adhesion, angiogenesis, and antigen presentation. We have shown recently that the proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human myeloma cells in vitro. In this study, we examined the efficacy, toxicity, and in vivo mechanism of action of PS-341 using a human plasmacytoma xenograft mouse model. One hundred immunodeficient (beige-nude-xid) mice were used in two independent experiments. The mice were injected s.c. with 3 × 10^7 RPMI-8226 myeloma cells. When tumors became measurable (9.2 days; range, 6–13 days after tumor injection), mice were assigned to treatment groups receiving PS-341 0.05 mg/kg (n = 13), 0.1 mg/kg (n = 15), 0.5 mg/kg (n = 14), or 1.0 mg/kg (n = 14) twice weekly via tail vein, or to control groups (n = 13) receiving the vehicle only. Significant inhibition of tumor growth, even with some complete tumor regression, was observed in PS-341-treated mice. The median overall survival was also significantly prolonged compared with controls (30 and 34 days for high-dose-treated mice versus 14 days for controls; P < 0.0001). PS-341 was well tolerated up to 0.5 mg/kg, but some mice treated at 1.0 mg/kg became moribund and lost weight. Analysis of tumors harvested from treated animals showed that PS-341 induced apoptosis and decreased angiogenesis in vivo. These studies therefore demonstrate that PS-341 has significant in vivo antmyeloma activity at doses that are well tolerated in a murine model, confirming our in vitro data and further supporting the early clinical promise of PS-341 to overcome drug resistance and improve patient outcome.

INTRODUCTION

The proteasome is a large intracellular molecule with multicatalytic protease activities found both in the cytoplasm and the nucleus (1, 2). It is an essential enzyme complex for the nonlysosomal, ATP-dependent proteolytic pathway (3), catalyzing the rapid degradation of intracellular proteins regulating cell cycle (4), apoptosis (5), cell adhesion (6), transcription (7), angiogenesis (8), and antigen presentation by MHC class I molecules (9). It is also essential for the rapid elimination of abnormal proteins, arising via mutation or by posttranslational damage such as oxidation. The catalytic core of this complex is found on the 20S proteasome subunit, a multicatalytic protease containing at least three peptidase activities: chymotryptic-like, tryptic-like, and post-glutamyl peptide hydrolyzing activities (10, 11). Proteasome inhibitors represent a potential active novel anticancer therapy (12, 13). They have been shown recently to inhibit proliferation and induce apoptosis of tumor cells in vitro. In xenograft murine models, proteasome inhibitors, alone or in combination, also induce tumor regression in human lymphoma (14) and carcinoma (15–17). PS-341, a dipetide boronic acid analogue, is a reversible proteasome inhibitor that selectively and specifically inhibits chymotryptic activity of the proteasome 20S subunit, presumably through its noncovalent binding to the NH2-terminal threonine residue containing a catalytic site (18).

We have shown recently that PS-341 acts both on MM4 cells and the bone marrow microenvironment to overcome drug resistance. Specifically, PS-341 inhibits MAP kinase signaling and MM cell proliferation; triggers caspase-3-mediated apoptosis; and overcomes drug resistance by inhibiting NF-κB, with resultant decreased adhesion molecule expression and MM cell binding to bone marrow stromal cells, as well as abrogation of related IL-6 secretion from bone marrow stromal cells (19). In this study, we examined the in vivo dose-response effects of PS-341 on tumor cell growth and survival in a human plasmacytoma xenograft murine model and characterized mechanisms of its in vivo antitumor activity.

MATERIALS AND METHODS

Animals. Six-week-old male triple immune-deficient BNX mice (n = 100) were obtained from Frederick Cancer Research and Developmental Center (Frederick, MD). They were maintained in a specific pathogen-free area in our animal resources facility. All animal studies were conducted according to protocols approved by the Animal Ethics Committee of the Dana-Farber Cancer Institute. They all received antibiotic (Enrofloxacin) as prophylaxis against infection.

Cells and Culture Conditions. The RPMI 8226 MM cell line (American Type Culture Collection) was cultured in RPMI 1640 containing 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 2 mm l-glutamine (Life Technologies, Inc.), 100 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies, Inc.). Before s.c. tumor cell injection into mice, cells were washed twice and resuspended in RPMI 1640 at a concentration of 3 × 10^7 cells/100 μl.

Drug. PS-341 (Millennium Pharmaceuticals, Inc.) mixed with mannitol (in a ratio of 1:10 to increase the solubility) was solubilized in 0.9% sodium chloride (Abbott Laboratories, North Chicago, IL) at the appropriate concentration before each drug injection.

Xenograft Murine Model. Mice were inoculated s.c. into the right flank with 3 × 10^7 MM cells in 100 μl of RPMI 1640, together with 100 μl of Matrigel basement membrane matrix (Becton Dickinson, Bedford, MA). When tumor was measurable, mice were assigned into four treatment groups receiving PS-341 or into a control group. Treatment with PS-341 was given i.v. twice weekly via tail vein at 0.05, 0.1, 0.5, and 1.0 mg/kg for 4 weeks. Subsequently, it was administered once weekly. The control group received the vehicle alone (0.9% sodium chloride) at the same schedule. Caliper measurements of the longest perpendicular tumor diameters were performed every alternate day to estimate the tumor volume, using the following formula: 4π/3 × (width/2)² × (length/2), representing the three-dimensional volume of an ellipse. Animals were sacrificed when their tumors reached 2 cm or when the mice...
became moribund. Survival was evaluated from the first day of treatment until death.

**Proteasome Inhibition Assay.** At the time of mouse sacrifice, 1 h after the last drug injection, tumors were excised for cell cycle profile study. Tumors were minced to a single cell suspension using a 70-µm cell strainer (Becton Dickinson, Franklin Lakes, NJ). Cells were first separated by Ficol-Hypaque density sedimentation, washed in PBS, and then fixed in 70% ethanol and treated with 10 µg/ml RNase (Roche Diagnostics Corp., Indianapolis, IN). Cells were then stained with propidium iodide (Sigma), and cell cycle profile was determined by flow cytometry using Coulter EPICS XL-MCL. Data were analyzed using the Phoenix flow system.

**Histopathology.** At the time of sacrifice, tumors were excised from mice, fixed in 10% neutral buffered formalin, and embedded in paraffin according to standard histological procedures. For MVD assays, 5-µm paraffin sections were used for immunohistochemistry for mouse CD34 expression. Slides were deparaffinized and pretreated with 1.0 mM EDTA, pH 8.0 (Zymed, South San Francisco, CA) in a steam pressure cooker (Decloaking chamber; BioCare Medical, Walnut Creek, CA), followed by washing in distilled water. All further steps were performed at room temperature in a hydrated chamber. Slides were treated with peroxidase block (DAKO, Carpinteria, CA) for 5 min to quench endogenous peroxidase activity, followed by a 1:5 dilution of goat serum in 50 mM Tris-Cl (pH 7.4), for 20 min to block nonspecific binding sites. Primary rat antimurine CD34 antibody (PharMingen, San Diego, CA) was applied at 1:100 dilution in 50 mM Tris-Cl (pH 7.4) with 3% goat serum for 1 h. After washing in 50 mM Tris-Cl (pH 7.4), secondary rabbit antirat antibody (DAKO) was applied at 1:200 dilution in 50 mM Tris-Cl (pH 7.4) with 3% goat serum for 30 min. Slides were washed again in 50 mM Tris-Cl (pH 7.4), and goat antirabbit horseradish peroxidase-conjugated antibody (Envision detection kit; DAKO) was applied for 30 min. After further washing, immunoperoxidase staining was developed using a 3,3′-diaminobenzidine chromogen kit (DAKO) and counterstained with hematoxylin.

MVD was determined by light microscopy, according to the procedure of Weidner et al. (21), without knowledge of the treatment history. Areas of most intense neovascularization were identified by scanning tumor sections at low power magnification (×40) and then counted at high power magnification (×400). At least five separate ×400 fields were analyzed by two investigators in a blinded fashion, using double-headed light microscopy.

The TUNEL assay was performed using the ApopTag kit (Intergen Co., Purchase, NY) on histological specimens fixed in formalin. Nucleotides contained in the reaction buffer were enzymatically added to the DNA using terminal deoxynucleotidyl transferase. Terminal deoxynucleotidyl transferase catalyzed the addition of nucleotide triphosphates to the 3′-OH ends of double-stranded or single-stranded DNA from apoptotic cells. The incorporated nucleotides formed an oligomer composed of digoxigenin and unlabeled nucleotide. DNA fragments labeled with the digoxigenin nucleotide were then detected by an antidigoxigenin antibody conjugated to peroxidase, allowing the formation of chromogenic substrates visible by light microscopy. H&E staining was also performed on tumor, liver, spleen, bone marrow, heart, lungs, gut, brain, and kidneys and studied for signs of toxicity using light microscopy.

**Statistical Analysis.** Two experiments were conducted to investigate the rate of tumor growth in the presence of PS-341. Each experiment included a group of control mice, as well as mice treated at two different doses of PS-341. Each experiment was analyzed separately. For tumor growth, diagnostic plots suggested that models of exponential growth were appropriate to the data. For each experiment, linear mixed-effect models were fit to the natural logarithm of tumor volume over time. This permits the estimation of an exponential parameter governing the rate of growth for each of the three groups of animals in each experiment. Tumor volume was measured with a calliper. Each animal within a group was treated with only one dose of the drug. Each treatment group was compared with the control, and in each of the experiments, the two treated groups were compared with one another. Overall survival in each experiment was measured using the Kaplan-Meier method, and results are presented as the median overall survival, with 95% CIs. Statistics for the cell cycle and protein expression were obtained using Fisher’s Exact Probability. Unpaired Student’s t tests were used to calculate the statistical significance of observed differences in MVD between treated and control groups; results are presented as mean ± SE.

**RESULTS**

**PS-341 Decreases Tumor Growth.** Two independent experiments, each using 50 mice, evaluated the effect of escalating doses of PS-341 on tumor cell growth and host survival. The first experiment examined the effect of low doses (0.05 and 0.1 mg/kg), and the second experiment studied high doses (0.5 mg/kg and 1.0 mg/kg). Each study had its own independent control cohort treated with the vehicle alone (0.9% normal saline). Mice were inoculated s.c. in the right flank with 3 × 10⁷ RPMI 8226 MM cells, and 82% animals developed a measurable tumor after a mean of 9.2 (6 to 13) days. The average tumor volume was 132.9 mm³ (159.7 mm³ in the first study and 106.0 mm³ in the second) at the beginning of treatment. Mice were then assigned to PS-341 treatment groups [0.05 mg/kg (n = 13), 0.1 mg/kg (n = 15), 0.5 mg/kg (n = 14), or 1.0 mg/kg (n = 14)] or 0.9% normal saline-treated control groups (n = 13 in both experiments). As shown in Fig. 1A, animals treated at the two lowest PS-341 doses (0.05 and 0.1 mg/kg) showed inhibition of tumor growth compared with controls, although this inhibition was not statistically significant in our model. However, statistically significant inhibition of tumor growth was observed in mice treated with PS-341 at 0.5 and 1.0 mg/kg versus the control group (P = 0.02 and P = 0.01, respectively; Fig. 1B). In a log-linear model, the daily growth rates were 0.22 for the control mice versus 0.07 and 0.06 for the mice treated with 0.5 and 1.0 mg/kg PS-341, respectively. Of note, tumor growth in half of the animals treated with 1.0 mg/kg was completely suppressed for a mean of 9 days.
PS-341 INHIBITION ON TUMOR GROWTH IN VIVO

PS-341 Prolongs Survival. As shown on Fig. 2A and as expected from tumor growth results, there was no statistically significant difference in the median overall survival of low-dose PS-341-treated mice versus control animals (P = 0.11). In contrast, a statistically significant prolongation in median overall survival was observed in animals treated with 0.5 mg/kg (P < 0.0001) and 1.0 mg/kg (P < 0.0001) PS-341 versus control animals; median overall survival was 14 days (95% CI, 12–14 days) in the control cohort versus 30 days (95% CI, 21–36 days) and 34 days (95% CI, 24–44 days) in groups treated with 0.5 and 1.0 mg/kg, respectively (Fig. 2B). There was also a trend toward prolonged survival in mice treated with 1.0 mg/kg PS-341 versus animals in the 0.5 mg/kg treatment group (P = 0.08).

PS-341 Induces Apoptosis. Tumor cells and tumor sections harvested from mice treated with 0.5 and 1.0 mg/kg PS-341 showed a statistically significant increase in apoptosis versus controls, as evidenced by cell cycle analysis (Fig. 3, A and B) and TUNEL assays (Fig. 3, C and D). Cell cycle analysis was performed on 33 tumors, 22 from mice treated at 0.5 and 1.0 mg/kg PS-341, and 11 from control animals. Apoptosis, as represented by the sub-G1 fraction (Fig. 3, C), was positive in 16 of 22 (73%) tumors examined from PS-341-treated animals versus 1 of 11 (9%) tumors from control animals (P < 0.001, Fisher’s Exact Probability).

PS-341 Decreases Blood Vessel Density in Tumors. Tumor sections from mice treated with 0.5 and 1.0 mg/kg PS-341, as well as from control mice (n = 5 in each group), were evaluated for MVD by immunohistochemical analysis for CD34 expression (Figs. 4, A and B). As shown in Fig. 4C, there was a statistically significant decrease in MVD in PS-341-treated tumors compared with control tumors: 16.4 ± 2.4 blood vessels/HPF (×400) for controls versus 7.4 ± 1.7 and 6.4 ± 2.1 for the mice treated with 0.5 and 1.0 mg/kg PS-341, respectively (P = 0.0001). However, there was no statistically significant difference in MVD in tumors from animals treated with 0.5 versus 1.0 mg/kg PS-341 (P = 0.4).

Proteasome Inhibition Assay. To determine the effect of PS-341 treatment on inhibition of 20S proteasome, tumor, whole blood, spleen, and liver from treated and control mice were collected and processed 1 h after the last treatment. PS-341 induced a dose-dependent decrease in proteasome activity in blood, liver, and spleen (Fig. 5A). The proteasome inhibition by PS-341 (1.0 mg/kg) in normal tissues correlates with the toxicity in mice. Importantly, PS-341 also

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Fig. 2. Effect of PS-341 on survival. BNX mice were inoculated with 3 × 10^7 RPMI 8226 cells s.c. in the right flank. i.v. twice weekly treatment was started on day 0, when tumors were measurable. Survival was evaluated from the first day of treatment until death; mice were sacrificed when their tumor diameters reached 2 cm or when they became moribund. A, mice were treated with PS-341 at 0.1 mg/kg (n = 15; ---) or 0.05 mg/kg (n = 13; ---), or with 0.9% sodium chloride vehicle only (n = 13; --). B, mice were treated with PS-341 at 1.0 mg/kg (n = 14; ---), 0.5 mg/kg (n = 14; ---), or with vehicle only (n = 13; ---).

Fig. 3. PS-341 induces apoptosis in vivo. Mice were sacrificed 1 h after the last treatment, and tumors were excised for evaluation of cell cycle profile and apoptosis using a TUNEL assay. For cell cycle analysis, tumors were minced to a single-cell suspension, and mononuclear cells were obtained by Ficoll-Hypaque density sedimentation. After cell permeabilization and RNase treatment, cells were stained with propidium iodide, and cell cycle profile was determined using flow cytometry. A, cell cycle profile of tumor harvested from a control mouse. B, cell cycle profile of tumor harvested from a mouse treated with 1 mg/kg PS-341. For TUNEL assay, ApopTag kit was used to examine histological specimens fixed in formalin. C, tumor section from a control mouse tumor at ×400. D, tumor section from a mouse treated with 1 mg/kg PS-341 at ×400.
induced a dose-dependent inhibition of proteasome function in tumors (Fig. 5B).

**Toxicity.** PS-341 was well tolerated in mice at doses up to 0.5 mg/kg, except for slightly less weight gain than in other groups (data not shown). In contrast, at the highest PS-341 dose (1.0 mg/kg), 2 of 14 (14%) mice became moribund and had to be sacrificed, and 3 (21%) mice lost >10% of their initial body weight; other mice treated at this high dose also did not gain weight normally. H&E staining on histological tissue sections was performed in eight PS-341-treated mice and 9 control mice. Although there was no evidence of toxicity in the liver, kidneys, heart, lungs, or other vital organs, bone marrow histology showed hypoplasia in 37% of mice treated with PS-341 at 1.0 mg/kg compared with no hypoplasia in control mice ($P = 0.08$, Fisher’s exact probability). However, peripheral blood was also examined, and no reduction in cell counts was seen in PS-341-treated mice compared with control mice (data not shown).

**DISCUSSION**

Our laboratory has previously published in vitro data demonstrating the potent effects of PS-341 on myeloma cell lines, as well as on primary patient myeloma cells (19). We have now demonstrated the striking dose-dependent efficacy of PS-341 against a human MM cell line using a recently published in vivo murine xenograft model (22). Significant inhibition of tumor growth and prolongation of survival was seen in PS-341-treated compared with control mice. To investigate the in vivo effects of PS-341, cell cycle analysis was performed on tumors excised and minced into single-cell suspension. In contrast to the G2-M arrest demonstrated previously in vitro, we did not observe G2-M arrest ex vivo. There was, however, a significant increase in the sub-G1 fraction representing apoptosis, which was confirmed with TUNEL assay performed on histological tumor sections. Because vascular endothelial growth factor production induces angiogenesis as well as myeloma cell proliferation and migration (23), we assessed MVD on histological sections using antimouse CD34 immunohistochemistry. Our model is particularly suited for studies of neoangiogenesis, with the proangiogenic basement membrane preparation Matrigel used to increase tumorigenicity at the site of tumor cell inoculation. Our results confirmed the antiangiogenic properties of PS-341 demonstrated by Sunwoo et al. (16) and Oikawa et al. (8).

Using the colorimetric proteasome inhibition assay (20), our study demonstrated dose-dependent proteasome inhibition in RBCs, liver, and spleen, as well as in tumor cells. The degree of proteasome inhibition was greater in normal tissues, despite minimal toxicity, confirming the increased sensitivity of myeloma cells versus normal cells to proteasome inhibition shown in our previous studies (19).

The transcription factor NF-kB in myeloma cells regulates drug resistance in tumor cells and adhesion molecule expression on tumor cells and bone marrow stromal cells and related binding, as well as transcription and secretion of myeloma growth and survival factors in the BM milieu (24). Our laboratory has previously shown inhibition of NF-kB.
activation in myeloma cells by PS-341, using the nuclear supershift EMSA assay (19). Sunwoo et al. (16) similarly demonstrated inhibition of NF-κB activation in squamous cell carcinoma cells by PS-341, with significant antitumor activity. Furthermore, Tan and Waldeman (25) showed that PS-341 decreased NF-κB DNA binding activity in adult T-cell leukemia cells by preventing IκBα degradation. Although PS-341 alone did not prolong survival of adult T-cell leukemia-tumor bearing mice, it did add to the effect of the humanized anti-IL-2Rα. Cusack et al. (26) also showed synergy of PS-341 with the topoisomerase I inhibitor CPT-11, evidenced by inhibition of SN38-induced NF-κB activity. These studies suggest that inhibition of NF-κB plays a role in the antitumor effects of PS-341; however, recent studies from our laboratory using a specific IκB kinase (IKK) inhibitor to selectively block NF-κB activation have shown only partial inhibition of myeloma cell survival (27). Interestingly, growth of myeloma cells adherent to bone marrow stromal cells, as well as IL-6 secretion triggered by this binding, was completely blocked using this IKK inhibitor. These studies both validate NF-κB as a therapeutic target and suggest that the antitumor effects of PS-341 are broader than selective NF-κB blockade. Ongoing gene array and proteomic studies are defining the molecular sequelae of PS-341 in myeloma cells to define mechanisms of drug sensitivity versus resistance. Our preliminary in vitro studies suggest that PS-341 down-regulates growth and survival kinases, induces apoptotic cascades, inhibits the proteasome-ubiquitin pathway, and induces stress responses. Importantly, the current animal study confirms that PS-341 induces tumor cell apoptosis, suppression of neoangiogenesis, and dose-dependent proteasome inhibition in vivo. Using this model, gene profiling and proteomic studies will similarly define in vivo targets of PS-341 and allow for the development of more potent targeted and less toxic therapeutics.

PS-341 has recently been evaluated in a Phase II trial as treatment of patients with relapsed refractory myeloma (28). Preliminary analysis in 54 patients who completed a minimum of two cycles of therapy showed that PS-341 induced either stabilization of disease or a reduction in myeloma paraprotein in 46 (85%) patients, with a favorable toxicity profile. Future studies will evaluate PS-341 treatment for patients with myeloma earlier in their disease course, as initial treatment or treatment of first relapse. Moreover, a Phase III trial is planned to compare PS-341 versus dexamethasone treatment for relapsed myeloma. In addition, our preclinical studies demonstrate that PS-341 has enhanced antitumor activity when combined with conventional (dexamethasone) or novel (immunomodulatory derivatives of thalidomide, IMiD) therapies, providing the framework for clinical trials of combination therapies.

PS-341, therefore, represents a novel class of myeloma therapy and is the first proteasome inhibitor to be used in clinical trials. Numerous animal studies have shown efficacy of PS-341 in various tumor cell lines, and synergistic antitumor activity has been demonstrated for PS-341 in combination with other chemotherapy. The current study provides strong in vivo evidence supporting use of PS-341 therapy in patients with multiple myeloma and will serve as a model both to evaluate potential combination therapies, as well as for delineating the in vivo molecular mechanism of antitumor activity of PS-341, ultimately allowing for the development of next-generation, more potent and targeted therapies.

ACKNOWLEDGMENTS

We thank Dr. Roderick T. Bronson at the Rodent Histopathology Core, Dana-Farber/Harvard Cancer Center, for mouse histopathological studies and Dr. Jeffery L. Kukut at the Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, for TUNEL assays and MVD evaluations.

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