

# The Proteasome Inhibitor PS-341 Inhibits Growth, Induces Apoptosis, and Overcomes Drug Resistance in Human Multiple Myeloma Cells<sup>1</sup>

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## ABSTRACT

Human multiple myeloma (MM) is a presently incurable hematological malignancy, and novel biologically based therapies are urgently needed. Proteasome inhibitors represent a novel potential anticancer therapy. In this study, we demonstrate that the proteasome inhibitor PS-341 directly inhibits proliferation and induces apoptosis of human MM cell lines and freshly isolated patient MM cells; inhibits mitogen-activated protein kinase growth signaling in MM cells; induces apoptosis despite induction of p21 and p27 in both p53 wild-type and p53 mutant MM cells; overcomes drug resistance; adds to the anti-MM activity of dexamethasone; and overcomes the resistance to apoptosis in MM cells conferred by interleukin-6. PS-341 also inhibits the paracrine growth of human MM cells by decreasing their adherence to bone marrow stromal cells (BMSCs) and related nuclear factor  $\kappa$ B-dependent induction of interleukin-6 secretion in BMSCs, as well as inhibiting proliferation and growth signaling of residual adherent MM cells. These data, therefore, demonstrate that PS-341 both acts directly on MM cells and alters cellular interactions and cytokine secretion in the BM milieu to inhibit tumor cell growth, induce apoptosis, and overcome drug resistance. Given the acceptable animal and human toxicity profile of PS-341, these studies provide the framework for clinical evaluation of PS-341 to improve outcome for patients with this universally fatal hematological malignancy.

## INTRODUCTION

Proteasome inhibitors represent potential novel anticancer therapy (1, 2). These agents inhibit the degradation of multiubiquitinated target proteins, *i.e.*, cell cycle regulatory proteins such as cyclins and cyclin-dependent kinase inhibitors, and regulate cell cycle progression (3). Proteasome inhibitors induce apoptosis of tumor cells, despite the accumulation of p21 and p27 and irrespective of the p53 wild-type or mutant status (4, 5). Accumulation of Bax induced by proteasome inhibitors can overcome the survival effect of Bcl-2 and increase cytochrome *c*-dependent apoptosis (6). Importantly, proteasome inhibitors can also overcome NF- $\kappa$ B activation and related drug resistance by inhibiting degradation of I $\kappa$ B and the P105 precursor of p50 subunit of NF- $\kappa$ B (7–10). Moreover, proteasome inhibitors are synergistic with Dex<sup>3</sup> in an asthma model (11). Finally, the proteasome inhibitor PS-341 demonstrated marked *in vivo* activity against human prostate cancer (1) and Burkitt's lymphoma (12) in a murine model;

produced additive growth delays with 5-fluorouracil, cisplatin, Taxol, and Adriamycin against Lewis lung carcinoma (13); and demonstrated antiangiogenic activity in an orthotopic pancreatic cancer model (14). PS-341 is nearly completing Phase I testing in humans, with an acceptable toxicity profile, and will soon be evaluated for efficacy in Phase II clinical trials.

MM is an incurable hematological malignancy, which affected 13,700 new individuals in the United States in 2000 (15), and novel biologically based therapies are, therefore, urgently needed. There are several characteristics of MM that suggest that it is an ideal candidate for proteasome inhibitor therapy. First, MM cells adhere to BMSCs, which both localizes them in the BM milieu (16) and confers resistance to apoptosis (17). Proteasome inhibitors have been reported to down-regulate cytokine-induced expression of VCAM-1 (18), a major ligand on BMSCs for VLA-4 on MM cells (19), and thereby might inhibit MM cell-BMSC binding and related protection against apoptosis. Second, adherence of MM cells to BMSCs triggers NF- $\kappa$ B-dependent transcription and secretion of IL-6 (19), a MM cell growth and survival factor (20). By virtue of its inhibition of NF- $\kappa$ B activation (7–10), PS-341 can inhibit this synthesis of IL-6. Third, Dex is a major therapy for MM, and PS-341 synergizes with Dex (11). Moreover, resistance to Dex in MM cells is conferred by IL-6 (21–23), and PS-341 may overcome Dex resistance by virtue of its effects on IL-6. In addition, NF- $\kappa$ B has been shown to play a role in the rescue of MM cells from Dex-induced apoptosis by Bcl-2 (24), and PS-341 may also overcome Dex resistance by inhibiting NF- $\kappa$ B activation. Finally, increased angiogenesis has recently been described in MM BM (25), as well as significant *in vitro* and clinical activity of antiangiogenic agents such as thalidomide and its analogues (26, 27). The antiangiogenic effect of PS-341 (14, 28), therefore, represents another potential mechanism of anti-MM activity.

In this study, we examined the effects of PS-341 on human MM cell lines, freshly isolated patient MM cells, as well as MM cells adherent to BMSCs. Given that PS-341 has a favorable toxicity profile, these studies provide the framework for clinical evaluation of PS-341 in patients with MM.

## MATERIALS AND METHODS

**MM-derived Cell Lines and Patient MM Cells.** Dex-sensitive (MM.1S) and Dex-resistant (MM.1R) human MM cell lines were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). Dox-, Mit-, and Mel-resistant (Dox40 cells, MR20 cells, and LR5 cells, respectively) RPMI8226 human MM cells were kindly provided by Dr. William Dalton (Moffitt Cancer Center, Tampa, FL). IM-9, U266, ARH-77, and Hs Sultanon cells were obtained from American Type Culture Collection (Rockville, MD). All of the MM cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO),  $2 \times 10^{-3}$  M L-glutamine, 100 units/ml penicillin (Pen), and 100  $\mu$ g/ml streptomycin (Strep; Life Technologies, Inc., Grand Island, NY). Drug-resistant cell lines were cultured with Dox, Mit, Mel, or Dex to confirm their lack of drug sensitivity. Patient MM cells (96% CD38 positive/CD45RA negative) were purified from patient BM samples, as described previously (29).

**BMSC Cultures.** BM specimens were obtained from patients with MM. Mononuclear cells separated by Ficoll-Hipaque density sedimentation were

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<sup>3</sup> The abbreviations used are: Dex, dexamethasone; MM, multiple myeloma; BM, bone marrow; BMSC, BM stromal cell; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen-4; IL, interleukin; NF- $\kappa$ B, nuclear factor  $\kappa$ B; Dox, doxorubicin; Mit, mitoxantrone; Mel, melphalan; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide; PI, propidium iodide; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; Ab, antibody; MAPK, mitogen-activated protein kinase; STAT3, signal transducing and transcription 3; ERK2, extracellular signal-regulated kinase 2; TNF, tumor necrosis factor; EMSA, electrophoretic mobility shift analysis; PBMC, peripheral blood mononuclear cell; HUVEC, human umbilical vein endothelial cell.

used to establish long-term BM cultures, as described previously (27). When an adherent cell monolayer had developed, cells were harvested in HBSS containing 0.25% trypsin and 0.02% EDTA and were washed and collected by centrifugation.

**Proteasome Inhibitor.** PS-341<sup>1</sup> [pyrazylCONH(CHPh)CONH(CHisobutyl)B(OH)<sub>2</sub>; Millennium Predictive Medicine Inc., Cambridge, MA] was dissolved in DMSO and stored at -20°C until use. PS-341 was diluted in culture medium (0.0001–10 × 10<sup>-3</sup> M) immediately before use. PS-341 and control media contained <0.1% DMSO.

**DNA Synthesis.** Proliferation was measured as described previously (27). MM cells (3 × 10<sup>4</sup> cells/well) were incubated in 96-well culture plates (Costar, Cambridge, MA) in the presence of media, PS-341, and/or Dex or recombinant IL-6 (Genetics Institute, Cambridge, MA) for 48 h at 37°C. DNA synthesis was measured by [<sup>3</sup>H]thymidine (NEN Products, Boston MA) uptake. Cells were pulsed with [<sup>3</sup>H]thymidine (0.5 μCi/well) during the last 8 h of 48-h cultures. All of the experiments were performed in triplicate.

**Growth Inhibition Assay.** The inhibitory effect of PS-341 on MM and BMSC growth was assessed by measuring MTT dye absorbance of the cells. Cells from 48-h cultures were pulsed with 10 μl of 5 mg/ml MTT to each well for the last 4 h of 48-h cultures, followed by 100 μl of isopropanol containing 0.04 N HCl. Absorbance was measured at 570 nm using a spectrophotometer (Molecular Devices Corp., Sunnyvale CA).

**Cell Cycle Analysis.** MM cells and patient MM cells cultured for 0, 4, 6, 8, 12, and 16 h in PS-341 (0.01 × 10<sup>-6</sup> M) or control media were harvested, washed with PBS, fixed with 70% ethanol, and treated with 10 μg/ml RNase (Roche Diagnostics Corp., Indianapolis, IN). Cells were then stained with PI (Sigma; 5 μg/ml), and cell cycle profile was determined using the program M software on an Epics flow cytometer (Coulter Immunology, Hialeah, FL), as in prior studies (27).

**Assays of Apoptosis.** MM cells were cultured for 12 h at 37°C in the presence of PS-341 (0.01 × 10<sup>-6</sup> M). To assay for apoptosis, genomic DNA, extracted using a genomic DNA purification kit (Promega, Madison, WI) was electrophoresed on 2% agarose gel containing 5 μg/ml ethidium bromide and was analyzed under UV light for DNA fragmentation, as in prior studies (27). Additional assays of apoptosis included PI staining for the percentage of sub-G<sub>0</sub>/G<sub>1</sub> phase cells and caspase-3 cleavage, as in our prior studies (27).

**Immunoblotting.** MM cells were cultured with PS-341 and were harvested, washed, and lysed using lysis buffer [50 × 10<sup>-3</sup> M Tris-HCl (pH 7.4), 150 × 10<sup>-3</sup> M NaCl, 1% NP40, 5 × 10<sup>-3</sup> M EDTA, 5 × 10<sup>-3</sup> M NaF, 2 × 10<sup>-3</sup> M Na<sub>3</sub>VO<sub>4</sub>, 1 × 10<sup>-3</sup> M PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin]. For detection of p21, p27, Bcl-2, Bax, caspase-3, phospho-MAPK, phospho-STAT3, ERK2, or α-tubulin, cell lysates were subjected to SDS-PAGE, transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA), and immunoblotted with anti-p21, anti-p27, anti-Bcl-2, anti-Bax, anti-ERK2, anti-caspase-3 (Santa Cruz Biotechnology), or anti-α-tubulin (Sigma) Abs. To characterize the inhibition of growth signaling by PS-341, immunoblotting was also done with anti-phospho-specific MAPK or anti-phospho-specific STAT3 Abs (New England Biolabs, Beverly, MA).

**Assays of NF-κB Activation.** To analyze the effect of PS-341 on degradation of IκB induced by TNFα (R&D Systems) in MM.1S cells, MM.1S cells were pretreated with control media (0.05% DMSO) or PS-341 (5 × 10<sup>-6</sup> M) for 2 h. TNFα (5 ng/ml) was then added for the times indicated, and the cells were washed with PBS. Whole-cell extracts were prepared and analyzed by Western blotting using anti-IκBα Ab (Santa Cruz Biotechnology). The antigen-Ab complexes were visualized by ECL.

To assay for NF-κB activation in BMSCs, BMSCs were preincubated with PS-341 (5 × 10<sup>-6</sup> M for 1 h) before stimulation with TNFα (10 ng/ml) for 10, 20, or 30 min. Cells were then pelleted, resuspended in 400 μl of hypotonic lysis buffer A [20 × 10<sup>-3</sup> M HEPES (pH 7.9), 10 × 10<sup>-3</sup> M KCl, 1 × 10<sup>-3</sup> M EDTA, 0.2% Triton X-100, 1 × 10<sup>-3</sup> M Na<sub>3</sub>VO<sub>4</sub>, 5 × 10<sup>-3</sup> M NaF, 1 × 10<sup>-3</sup> M PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin], and kept on ice for 20 min. After centrifugation (14,000 × g for 5 min) at 4°C, the nuclear pellet was extracted with 100 μl of hypertonic lysis buffer B [20 × 10<sup>-3</sup> M HEPES (pH 7.9), 400 × 10<sup>-3</sup> M NaCl, 1 × 10<sup>-3</sup> M EDTA, 1 × 10<sup>-3</sup> M Na<sub>3</sub>VO<sub>4</sub>, 5 × 10<sup>-3</sup> M NaF, 1 × 10<sup>-3</sup> M PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin] on ice for 20 min. After centrifugation (14000 × g for 5 min) at 4°C, the supernatant was diluted to 100 × 10<sup>-3</sup> M NaCl and subjected to SDS-PAGE. Nuclear extracts were immunoblotted with anti-p65 NF-κB Ab (Santa Cruz Biotechnology). The PVDF mem-

brane was stripped and reprobed with anti-nucleolin Ab (Santa Cruz Biotechnology) to confirm equal loading of protein.

**EMSA.** Nuclear extracts for EMSAs were carried out as in our previous studies (19). Double-stranded NF-κB consensus oligonucleotide probe (5'-GGGGACTTCCC-3', Santa Cruz Biotechnology) was end-labeled with [γ-<sup>32</sup>P]ATP (50 μCi at 222 TBq/mM; NEN, Boston, MA). Binding reactions containing 1 ng of oligonucleotide and 3 μg of nuclear protein were conducted at room temperature for 20 min in total volume of 10 μl of binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol (v/v), and 0.5 μg of poly (dI/dC) (Pharmacia, Peapack, NJ)]. For supershift analysis, 1 μg of anti-p65 NF-κB Ab was added 5 min before the reaction mixtures, immediately after the addition of radiolabeled probe. The samples were loaded onto a 4% polyacrylamide gel, transferred to Whatman paper (Whatman International, Maidstone, United Kingdom), and visualized by autoradiography.

**Effect of PS-341 on Paracrine MM Cell Growth and Signaling in the BM.** Adhesion assays were performed as described previously (19). MM.1S cells were pretreated with PS-341 for 12 h, washed, and labeled with Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (NEN). Cells were then added to BMSC-coated 96-well plates and incubated for 1 h. After incubation, each well was washed twice with media and lysed with 0.5% NP40, and lysate radioactivity was counted on a gamma counter.

To evaluate growth stimulation and signaling in MM cells adherent to BMSCs, 3 × 10<sup>4</sup> MM.1S cells were cultured in BMSC-coated 96-well plates for 48 h in the presence or absence of PS-341. DNA synthesis was measured as described above. To characterize the signaling in MM cells that is triggered by the adhesion to BMSCs, MM.1S cells (5 × 10<sup>6</sup>) were cultured in BMSC-coated 6-well plates for 4 h in the presence or absence of PS-341 (5 × 10<sup>-6</sup> M). MM.1S cells were harvested, washed with PBS, lysed, subjected to SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-phospho-MAPK and anti-phospho-STAT3 Abs. The Duoset ELISA (R&D System) was used to measure IL-6 in supernatants of 48-h cultures of BMSCs with or without MM.1S cells, in the presence or absence of PS-341.

**Statistical Analysis.** Statistical significance of differences observed in drug-treated *versus* control cultures was determined using Student's *t* test. The minimal level of significance was *P* < 0.05.

## RESULTS

**Effect of PS-341 on Growth of MM Cell Lines and MM Patients' Cells.** We first determined the effect of PS-341 on growth of MM cell lines (U266, IM-9, and Hs Sultan) cultured for 48 h, either in the presence or absence of PS-341, using MTT assay. As shown in Fig. 1A, the growth of these MM cell lines was completely inhibited by PS-341 (0.1 × 10<sup>-6</sup> M). Fifty % growth inhibition (IC<sub>50</sub>) in U266, IM-9, and Hs Sultan cells was noted at concentrations of 0.003, 0.006, or 0.02 × 10<sup>-6</sup> M, respectively. To examine whether there was cross-resistance between PS-341 and conventional therapies, RPMI8226 MM cells resistant to Dox (Dox/40 cells), Mit (MR20 cells), and Mel (LR5 cells) were similarly studied. Proliferation of Dox40, MR20, and LR5 MM cells was unaffected by culturing with 400 nM/liter Dox, 20 nM/liter Mit, and 5 × 10<sup>-6</sup> M Mel, respectively (data not shown). Importantly, cell growth of Dox40, MR20, and LR5 MM cells was completely inhibited by PS-341 (0.1 × 10<sup>-6</sup> M; Fig. 1B). IC<sub>50</sub> of PS-341 in RPMI8226, Dox40, MR20, and LR5 was 0.03, 0.04, 0.02, and 0.02 × 10<sup>-6</sup> M, respectively. Dex-sensitive (MM.1S) and -resistant (MM.1R) MM cell lines were similarly examined. As can be seen in Fig. 1C, growth of both cell lines was completely inhibited by PS-341 (0.01 × 10<sup>-6</sup> M). IC<sub>50</sub> of PS-341 in MM.1S and MM.1R cells was 0.0015 and 0.003 × 10<sup>-6</sup> M, respectively. These data demonstrate that PS-341 effectively inhibits the growth of chemoresistant MM cells at pharmacologically achievable doses and can overcome resistance to Dox, Mit, Mel, and Dex. As was true for MM cell lines, cell growth of four patients' MM cells (MM1–4) was also inhibited by PS-341 in a dose-dependent fashion (Fig. 1D), with IC<sub>50</sub>

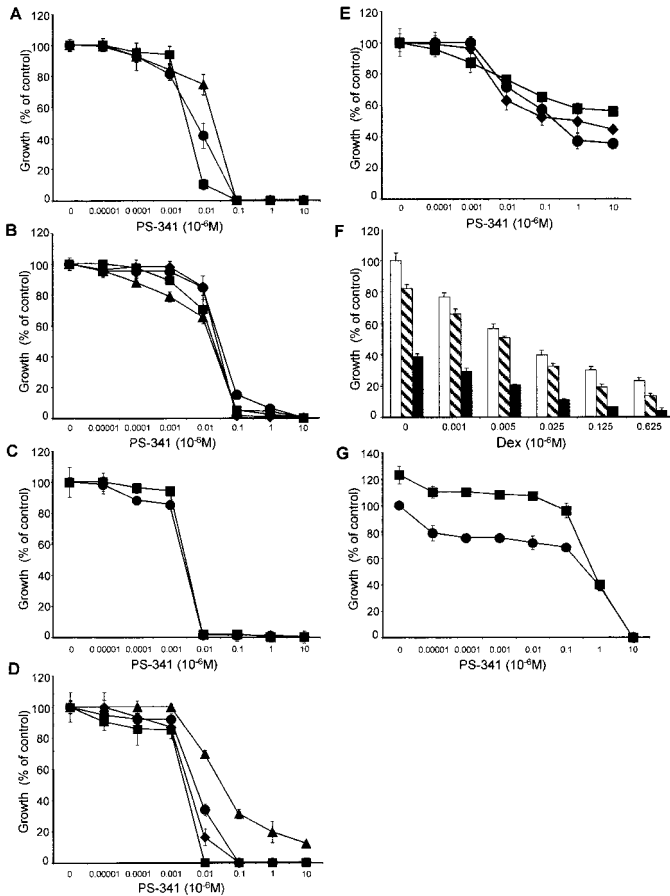


Fig. 1. Effect of PS-341 on growth of MM cell lines and patient MM cells. A, U266 (■), Hs Sultan (▲), and IM-9 (●) MM cells; B, drug-sensitive RPMI (◆), Dox-resistant (Dox40; ●), Mit-resistant (MR20; ▲), and Mel-resistant (LR5; ■) MM cells; C, Dex-sensitive MM.1S (●) and -resistant MM.1R (■) cells; and D, patient 1 (◆), 2 (●), 3 (▲), and 4 (■) MM cells were cultured with PS-341 (0.00001–10 × 10<sup>-6</sup> M). E, PBMCs from normal volunteers were cultured with PS-341 (0.0001–10 × 10<sup>-6</sup> M). F, MM.1S cells were cultured with 0.001–0.625 × 10<sup>-6</sup> M Dex in control media (□) and with PS-341 0.0025 (▨) or 0.005 (■) × 10<sup>-6</sup> M. G, MM.1S cells were cultured in control media alone or with PS-341 (0.00001–10 × 10<sup>-6</sup> M) in the presence (■) or absence (●) of IL-6 (50 ng/ml). In each case, cell growth was assessed by MTT assay. Values represent the mean (± SD) of triplicate cultures.

of PS-341 at 0.0035, 0.005, 0.03, and 0.0025 × 10<sup>-6</sup> M, respectively. PS-341 similarly inhibited the proliferation of these MM cell lines and patient MM cells, as assessed by [<sup>3</sup>H]thymidine uptake in 48-h cultures (data not shown). PBMCs from three normal volunteers were also examined for their susceptibility to PS-341. As can be seen in Fig. 1E, IC<sub>50</sub> of PS-341 in these PBMCs is ≥0.1 × 10<sup>-6</sup> M. These findings are consistent with the reported increased sensitivity to proteasome inhibitors of B chronic lymphocytic leukemia cells relative to normal B lymphocytes (30).

To determine whether PS-341 is additive with conventional therapies, we next examined the effect of Dex together with PS-341 on proliferation of Dex-sensitive MM.1S cells. As can be seen in Fig. 1F, MTT assay at 48 h revealed that PS-341 alone (0.0025 and 0.005 × 10<sup>-6</sup> M) and Dex alone (0.001 to 0.625 × 10<sup>-6</sup> M) each significantly inhibited MM.1S cell growth in a dose-dependent fashion and, furthermore, showed that their growth inhibitory effects are additive.

Given the additive effect of PS-341 and Dex (11), as well as the known role of IL-6 as a growth factor (31) and an inhibitor of Dex-induced apoptosis (32–34), we next examined whether exogenous IL-6 could overcome the growth inhibitory effect that is triggered by PS-341. Although IL-6 (50 ng/ml) triggered a 1.3-fold

increase in MM.1S cell growth in cultures with media alone, PS-341 inhibited this response in a dose-dependent fashion (Fig. 1G). This result showed that IL-6 does not overcome the inhibitory effect of PS-341 on MM cell growth.

**PS-341 Induces Apoptosis and IκBα Degradation and Inhibits p44/42 MAPK Activation in MM Cells.** To further analyze the mechanism of PS-341-induced inhibition of DNA synthesis and to determine whether PS-341 induced apoptosis of MM cells, we examined the cell cycle profile of U266 and patient MM cells cultured with media or PS-341 (0.01 × 10<sup>-6</sup> M) for 0, 4, 6, 8, 12, and 16 h. After incubation, cells were harvested and stained with PI. As shown in Fig. 2A, PS-341 induced a progressive increase in sub-G<sub>0</sub>/G<sub>1</sub> phase cells in a time-dependent manner; similar results were observed for RPMI8226 and MM.1S cells (data not shown). To confirm these results, we performed agarose gel electrophoresis using genomic DNA purified from MM cell lines and patient MM cells treated with PS-341 (0.01 × 10<sup>-6</sup> M) for 12 h. Apoptosis, evidenced by DNA fragmentation, was induced by PS-341 (Fig. 2B).

Apoptosis triggered by PS-341 was further confirmed by cleavage of caspase-3 in U266 cells (Fig. 2C) as well as RPMI8226, MM.1S, and patient MM cells (data not shown). Apoptosis occurred despite up-regulation of p21 and p27 in U266, IM9, ARH77, and RPMI8226 cells (Fig. 2C). No changes in Bcl-2 or Bax expression were induced by PS-341. These cell lines contain either wild-type p53 or mutant p53 phenotype, and these results confirm prior reports that proteasome inhibitors can induce apoptosis in both settings (1, 4, 5).

To determine whether the apoptotic effect of PS-341 is reversible, RPMI8226 cells were treated with 0.01 × 10<sup>-6</sup> M PS-341 for 0, 2, 4, 6, 8, 12, or 24 h. Cells were then washed and cultured in PS-341-free media for 24 h. Cell viability and percentage of apoptotic cells were assessed by trypan blue and PI staining, respectively. Fig. 2E demonstrates an irreversible progressive drug exposure time-dependent effect of PS-341 on RPMI 8226 cells: ≥50% growth inhibition was observed in cultures with PS-341 for ≥6 h, with complete abrogation of growth at exposure times of >12 h.

Because PS-341 inhibits TNFα-stimulated activation of NF-κB in primary HUVECs by blocking the degradation of the inhibitor IκBα (33), we next examined whether PS-341 also inhibited degradation of IκBα in TNFα-treated MM cells. Specifically, MM.1S cells were treated with 5 × 10<sup>-6</sup> M PS-341 or control media for 1 h and were subsequently stimulated by TNFα (5 ng/ml). As can be seen in Fig. 2F, IκBα decreased after stimulation of TNFα in DMSO control media-treated MM.1S cells, but not in PS-341-treated cells. Inhibition of NF-κB activation by PS-341 was further confirmed by EMSA. As can be seen in Fig. 2G, activation of NF-κB by TNFα was inhibited by pretreatment with PS-341 (5 × 10<sup>-6</sup> M for 1 h). These data indicated that PS-341 inhibits NF-κB activation in MM cells by stabilizing IκBα.

Because we have shown that proliferation of MM cells induced by IL-6 is mediated via the Ras-Raf MAPK cascade (31), we also determined whether PS-341 inhibits the activation of p42/44 MAPK that is triggered by IL-6. As can be seen Fig. 2H, tyrosine phosphorylation of p42/44 MAPK that was triggered by IL-6 was inhibited completely by PS-341 pretreatment of MM.1S cells for 2 h, whereas the activation of STAT3 was unaffected. This result demonstrates that PS-341 selectively inhibits the tyrosine phosphorylation of MAPK that is triggered by IL-6 in MM.1S cells.

**Effect of PS-341 on Paracrine MM Cell Growth and Signaling in the BM Microenvironment.** We next examined the effect of PS-341 on paracrine MM cell growth and signaling in the BM. As shown in Fig. 3A, PS-341 inhibited the proliferation of two MM patients' BMSCs in a dose-dependent fashion, with IC<sub>50</sub> of 5 and

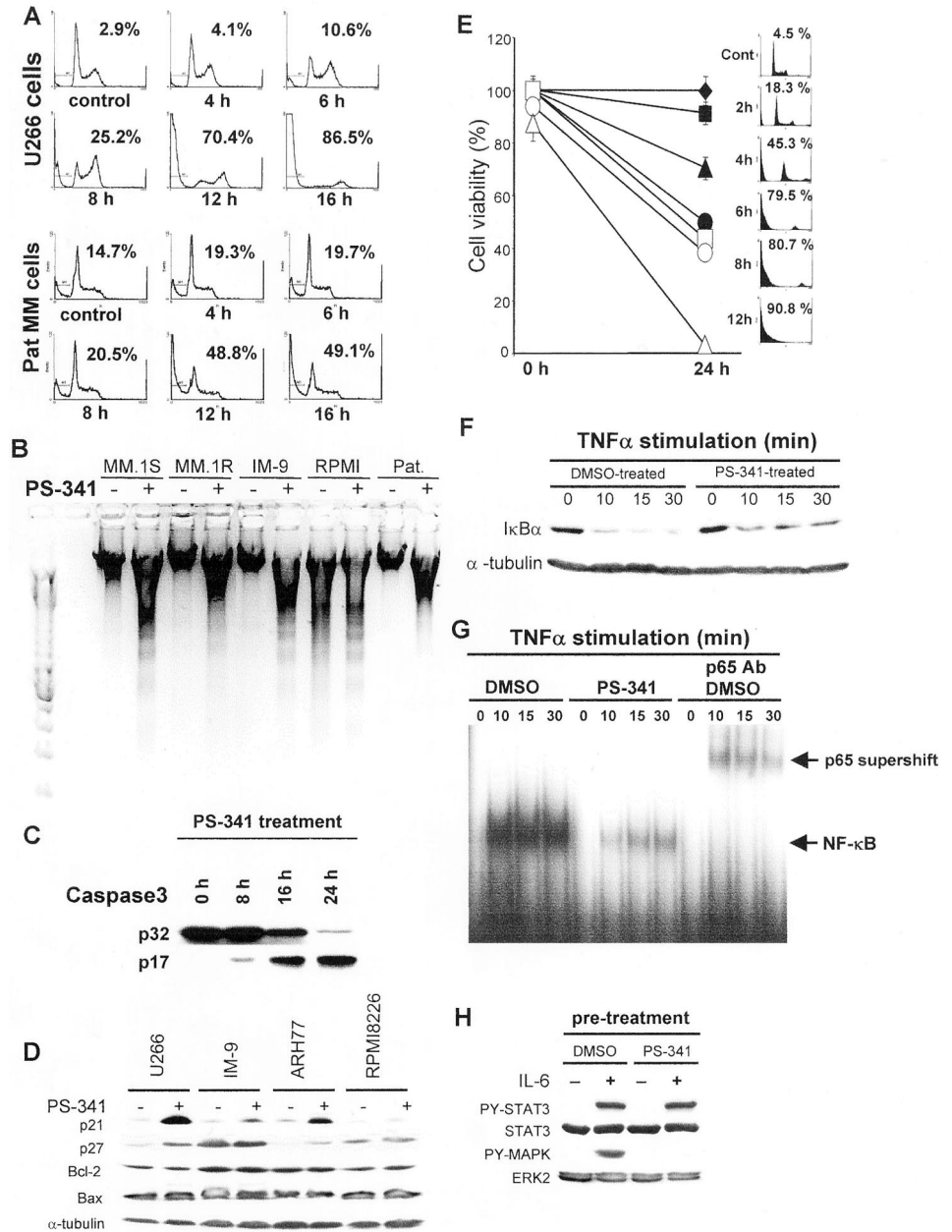


Fig. 2. Effect of PS-341 on cell cycle profile, apoptosis, and signaling cascades triggered by IL-6 in MM cell lines and patient MM cells. **A**, U266 and patient MM cells were treated with PS-341 ( $0.01 \times 10^{-6}$  M) for the time intervals indicated. Cell cycle profile was evaluated by PI staining using flow cytometry. % on each panel, percentage of sub-G<sub>0</sub>/G<sub>1</sub> cells. **B**, genomic DNA was extracted from MM.1S, MM.1R, IM-9, RPMI8226, and patient MM cells treated with either DMSO control (-) or PS-341 (+;  $0.01 \times 10^{-6}$  M for 12 h), and subjected to 2% agarose gel electrophoresis. **C**, U266 cells were incubated with PS-341 ( $0.01 \times 10^{-6}$  M), and cell lysates were immunoblotted with anti-caspase-3 Ab. **D**, lysates from U266, IM-9, ARH77, and RPMI8226 MM cells treated with control DMSO (-) or PS-341 (+;  $0.01 \times 10^{-6}$  M for 12 h) were immunoblotted with anti-p21, p27, Bcl-2, Bax, and  $\alpha$ -tubulin Abs. **E**, RPMI8226 cells were cultured with control media ( $\blacklozenge$ ) or  $0.1 \times 10^{-6}$  M PS-341 for 2 ( $\blacksquare$ ), 4 ( $\blacktriangle$ ), 6 ( $\bullet$ ), 8 ( $\square$ ), 12 ( $\circ$ ), and 24 ( $\triangle$ ) h. Cells were washed, resuspended in PS-341-free media, and then stained with trypan blue and PI to assess cell viability and apoptosis. **F**, MM.1S cells were pretreated with either DMSO control or PS-341 ( $5 \times 10^{-6}$  M for 1 h) prior to stimulation with TNF $\alpha$  (5 ng/ml) for indicated times. Whole-cell lysates were immunoblotted with anti-I $\kappa$ B $\alpha$  Ab to assess degradation of I $\kappa$ B $\alpha$ . **G**, MM.1S cells were pretreated with either DMSO control or PS-341 ( $5 \times 10^{-6}$  M for 1 h) prior to stimulation with TNF $\alpha$  (5 ng/ml) for indicated time intervals. EMSA using nuclear extract was performed as described in "Materials in Methods." **H**, MM.1S cells were pretreated with either DMSO control or PS-341 ( $5 \times 10^{-6}$  M for 1 h) before stimulation with IL-6 (50 ng/ml). Whole-cell lysates were immunoblotted with anti-phospho-STAT3, -phospho-MAPK, or -ERK2 Abs.

$10 \times 10^{-6}$  M, respectively. This IC<sub>50</sub> was more than 170-fold higher than for MM cell lines and patient MM cells.

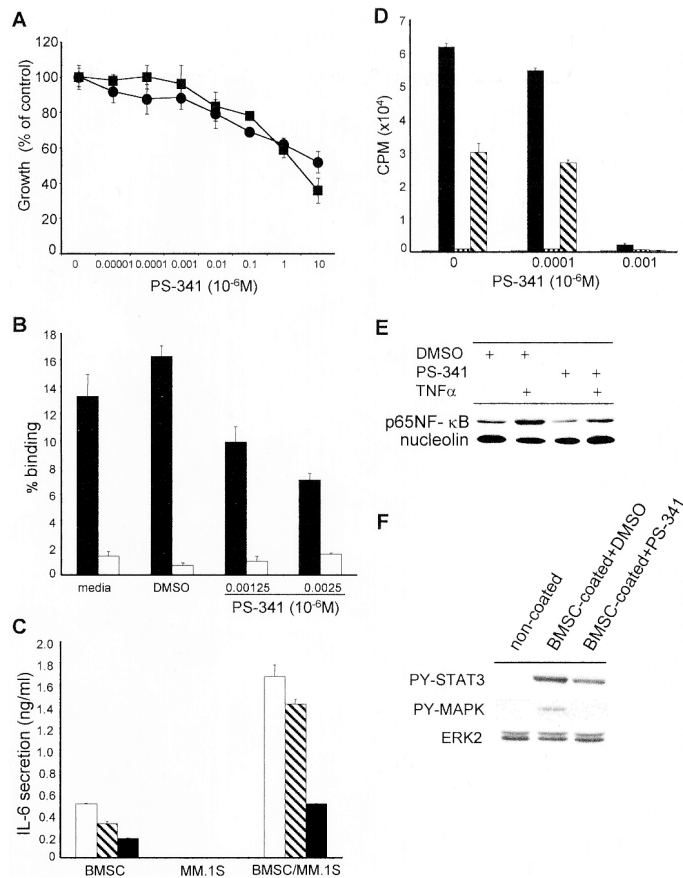
Adhesion molecules on MM cells mediate their binding to BMSCs, via VLA-4 on the MM surface binding to VCAM-1 on BMSCs, localizing them in the BM milieu (19). Given that PS-341 inhibits transcription of VCAM-1 and expression of VCAM-1 on HUVECs (18), we next determined whether PS-341 altered the adhesion of MM cells to BMSCs. Treatment with PS-341 decreased MM cell to BMSC binding by 50% (Fig. 3B). Binding of MM cells to BMSCs triggers NF- $\kappa$ B-dependent transcription and the secretion of IL-6 in BMSCs (19), which mediates paracrine growth and survival of tumor cells. As shown in Fig. 3C, the adhesion of MM.1S cells to BMSCs induced a >3-fold increase in IL-6 secretion, which was inhibited to baseline levels by PS-341 ( $0.01$  and  $0.1 \times 10^{-6}$  M). The binding of MM cells to BMSCs also triggered a 2-fold increase in DNA synthesis of MM.1S cells, which was completely abrogated by PS-341 ( $0.001 \times 10^{-6}$  M; Fig. 3D).

Given our prior studies, which showed that MM-cell binding to

BMSCs triggers NF- $\kappa$ B-dependent transcription and secretion of IL-6 in BMSCs, we next examined the effect of PS-341 on NF- $\kappa$ B activation in BMSCs, assessed by p65 NF- $\kappa$ B nuclear translocation. As can be seen in Fig. 3E, TNF $\alpha$  (10 ng/ml for 15 min) induced nuclear p65 NF- $\kappa$ B in BMSCs, and PS-341 blocked this response. Finally, PS-341 also blocked the activation of MAPK, but not of STAT3, in MM cells adherent to BMSCs for 4 h (Fig. 3F), consistent with its effect of inhibiting tumor cell growth and proliferation.

## DISCUSSION

Recent studies have shown that proteasome inhibitors represent potential novel anticancer therapy (1, 2.). These agents inhibit the degradation of multiubiquitinated target proteins, *i.e.*, cell cycle regulatory proteins such as cyclins and cyclin-dependent kinase inhibitors, and regulate cell cycle progression (3). In this study, we demonstrate that the proteasome inhibitor PS-341 not only inhibits growth and induces apoptosis of MM cell lines and patient MM cells resistant



**Fig. 3.** Effect of PS-341 on paracrine MM.1S and patient MM cell growth and signaling in the BM microenvironment. **A**, growth of MM patient 1 (●) and 2 (■) BMSCs in 48-h cultures with PS-341 (0.00001– $10 \times 10^{-6}$  M) was measured by MTT assay. **B**,  $^{51}\text{Cr}$ -labeled MM.1S cells were cultured with either BMSC-coated (■) or noncoated control (□) plates, and percentage of adhesion was assessed at 1 h. **C**, BMSCs or MM.1S cells were cultured alone or together in the absence (□) or presence of 0.01 (▨) or  $0.1 \times 10^{-6}$  M (■) PS-341; IL-6 was measured in supernatants of 48 h cultures by ELISA. **D**, BMSCs (□), MM.1S cells with BMSCs (■), MM.1S cells with 1% paraformaldehyde fixed BMSCs (▨), or MM.1S cells alone (▩) were cultured in the absence or presence of PS-341 (0.0001 and  $0.001 \times 10^{-6}$  M). [ $^3\text{H}$ ]Thymidine uptake was measured during the last 8 h of 48-h cultures. **E**, BMSCs were pretreated with PS-341 ( $5 \times 10^{-6}$  M for 1 h) and stimulated with TNF $\alpha$  (5 ng/ml). Nuclear translocation of p65-NF- $\kappa$ B was detected by the immunoblotting of nuclear extracts. PVDF membrane was stripped and reprobed with nucleolin. **F**, MM.1S cells were incubated in BMSC-coated plates for 4 h, either in the presence or absence of PS-341 ( $5 \times 10^{-6}$  M). MM.1S cells were harvested, lysed, electrophoresed, and immunoblotted with phospho-STAT3, phospho-MAPK, and ERK Abs.

to conventional therapies but also inhibits binding of MM cells to BMSCs with related up-regulation of IL-6 secretion and paracrine MM cell growth. Given the favorable toxicity profile of PS-341, these studies provide the framework for clinical trials of PS-341 in MM.

In this study, we first showed that PS-341 acts directly to inhibit the growth of MM cell lines and patient MM cells, assessed both by MTT assay and DNA synthesis. Growth inhibition of MM cell lines that were sensitive and resistant to Mel, Dox, and Dex was observed at an  $\text{IC}_{50}$  of  $<0.01 \times 10^{-6}$  M PS-341. These data demonstrate that PS-341 effectively inhibits the growth of chemoresistant MM cells at pharmacologically achievable doses and suggests independent mechanisms of resistance to Dox, Mel, and Dex *versus* PS-341. These results are also consistent with the reported increased sensitivity to proteasome inhibitors of B chronic lymphocytic leukemia cells relative to normal B lymphocytes (30).

Given the additive effect of PS-341 and Dex (11), as well as the known effect of IL-6 as a growth factor (31) and an inhibitor of Dex-induced apoptosis (23), we examined whether Dex added to the

anti-MM activity of PS-341; and conversely, whether exogenous IL-6 could abrogate its antitumor effects. Importantly, Dex enhanced the inhibitory effects of PS-341 on MM growth, evidenced by MTT assay; moreover, IL-6 cannot protect MM cells against PS-341. These studies suggest the potential therapeutic advantage of combined Dex and PS-341 in treatment protocols for MM.

Our studies further confirmed that PS-341 induced apoptosis of MM cells, evidenced by cell cycle analysis, DNA fragmentation, and caspase 3 cleavage. Apoptosis occurred despite the induction of p21 and p27, and without associated changes in Bcl-2 or Bax expression. Importantly, it occurred in MM cell lines containing either wild-type p53 or mutant p53, confirming prior reports that proteasome inhibitors can induce apoptosis in both settings (1, 4, 5). Moreover, the apoptotic effect of PS-341 was irreversible at all of the intervals examined (2–24 h), further suggesting its potential clinical utility.

In this study, PS-341 inhibited IL-6-triggered activation of p42/44 MAPK, known to mediate proliferation (31), as well as TNF- $\alpha$ -induced activation of NF- $\kappa$ B, known to mediate drug resistance (24). In contrast, the IL-6 induced activation of STAT3 in MM cells was unaffected by PS-341, demonstrating differential susceptibility of protein kinases in the Raf/MEK/MAPK *versus* JAK/STAT3 signaling pathways to PS-341.

Our study demonstrates the importance of studying the effects of novel agents such as proteasome inhibitors not only on the tumor cells directly, but also within the BM microenvironment. Importantly, these studies show that PS-341 also inhibited paracrine growth of MM cells within the BM milieu. First, adhesion of MM cells to BMSCs confers protection against apoptosis (17), and PS-341 inhibits tumor cell binding. Second, PS-341 inhibited MAPK growth signaling (31), even in those MM cells adhering to BMSCs, overcoming the growth-promoting effects of BMSC binding. Third, PS-341 abrogates the NF- $\kappa$ B-dependent up-regulation of IL-6 triggered by tumor to BMSC binding (19), which is of central importance given that IL-6 is the major growth and survival factor for MM cells (20). In previous studies, NF- $\kappa$ B activation conferred protection of tumor cells against apoptosis by modulating transcription targets of the Bcl-2 homologue Bfl/A1, the immediate-early response gene *IEX-1 L*, the inhibitors of apoptosis c-IAP1 and c-IAP2, and TNF receptor-associated factors 1 and 2 (8, 34, 35). These studies, coupled with our prior studies demonstrating that IL-6 can block Dex-induced apoptosis and confer drug resistance in MM cells (21, 23, 32), further suggest that the inhibition of NF- $\kappa$ B activation by PS-341 can overcome drug resistance.

These studies, therefore, demonstrate that the proteasome inhibitor PS-341 both directly induces apoptosis of human MM cells and abrogates paracrine growth of MM cells in the BM via altering cellular interactions and cytokine secretion in the BM milieu. They provide the framework for the clinical investigation of these novel agents to improve outcome for patients with this presently incurable disease.

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## The Proteasome Inhibitor PS-341 Inhibits Growth, Induces Apoptosis, and Overcomes Drug Resistance in Human Multiple Myeloma Cells

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