Molecular mechanisms mediating antimyeloma activity of proteasome inhibitor PS-341

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We have recently shown that proteasome inhibitor PS-341 induces apoptosis in drug-resistant multiple myeloma (MM) cells, inhibits binding of MM cells in the bone marrow microenvironment, and inhibits cytokines mediating MM cell growth, survival, drug resistance, and migration in vitro. PS-341 also inhibits human MM cell growth and prolongs survival in a SCID mouse model. Importantly, PS-341 has achieved remarkable clinical responses in patients with refractory relapsed MM. We here demonstrate molecular mechanisms whereby PS-341 mediates anti-MM activity by inducing p53 and MDM2 protein expression; inducing the phosphorylation (Ser15) of p53 protein; activating c-Jun NH2-terminal kinase (JNK), caspase-8, and caspase-3; and cleaving the DNA protein kinase catalytic subunit, ATM, and MDM2. Inhibition of JNK activity abrogates PS-341-induced MM cell death. These studies identify molecular targets of PS-341 and provide the rationale for the development of second-generation, more targeted therapies.

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Introduction

The ubiquitin-proteasome pathway (UPP) is a proteolytic system in the cytosol and nucleus that regulates cyclins and cyclin-dependent kinase inhibitor cell-cycle regulatory proteins and thereby regulates cell-cycle progression.1 UPP also has a critical role in the selective removal of mutant, damaged, and misfolded proteins. Proteasome inhibitors have recently demonstrated promise as potential novel anticancer therapies2 because they induce the apoptosis of tumor cells in vitro, despite the accumulation of p21 and p2714 and irrespective of the p53 wild-type or mutant status5,6 in tumor cells. Specifically, PS-341 (pyrazylcarbonyl-Phe-Leu-boronate) is representative of a class of peptide boronate proteasome inhibitors that inhibit 26S proteasome activity.1 This agent induces marked in vivo antitumor activity against human prostate cancer,2,7 Burkitt lymphoma in a murine model,8 and adult T-cell leukemia;9 produces—with 5-fluorouracil, cisplatin, paclitaxel (Taxol), and doxorubicin hydrochloride (Adriamycin)—additive growth delays against Lewis lung carcinoma10; and demonstrates antiangiogenic activity in an orthotopic pancreatic cancer model.11

Multiple myeloma (MM) is a hematologic malignancy that affected 14,000 new patients in the United States in 2000,12 and it remains incurable with conventional therapies. Novel biologically based therapies are, therefore, urgently needed. We have previously demonstrated that PS-341 directly induces apoptosis by the activation of caspase-3 and without the associated alteration of Bax or Bcl-2 protein expression, even in MM cell lines and patient MM cells that are resistant to conventional therapies. PS-341 also overcomes the antiapoptotic effects of interleukin-6 (IL-6) or adherence to bone marrow stromal cells (BMSCs).4 In vivo PS-341 inhibits human MM cell growth and associated angiogenesis and prolongs survival in a murine SCID mouse model. Most important, PS-341 has achieved responses, even complete responses, in a phase 2 clinical trial treating patients with relapsed MM refractory to conventional therapies, and it has an acceptable toxicity profile.13 This drug, therefore, represents a new treatment paradigm targeting not only the tumor cell but also the MM cell–host interaction and the bone marrow (BM) milieu to overcome drug resistance and improve patient outcome.

Both proapoptotic and antiapoptotic proteins are substrates of UPP1 and the molecular mechanisms whereby PS-341 mediates its anti-MM activity are not yet defined. In this study, we demonstrate that PS-341 induces p53 and MDM2 protein expression; induces phosphorylation (Ser15) of p53 protein; and activates c-Jun NH2-terminal kinase (JNK), which in turn activates caspase-8 and caspase-3. Activated caspase-3, in turn, cleaves DNA protein kinase catalytic subunit (DNA-PKcs), ATM, and MDM2; conversely, caspase inhibitors Z-VDAD-FMK and Z-IETD-FMK abrogate these effects. Furthermore, the inhibition of JNK activity by SP600125 reduces PS-341–induced MM cell death. Our results demonstrate that PS-341 induces caspase activation, inhibits DNA repair, and activates p53 by phosphorylation and degradation of MDM2. Given the early clinical promise and favorable toxicity profile of PS-341 in patients with relapsed refractory MM, these studies provide the framework for further clinical evaluation of PS-341, alone and coupled with conventional or other novel therapies, to improve patient outcome in MM.

Materials and methods

MM-derived cell lines and patient MM cells

Dr Steven Rosen (Northwestern University, Chicago, IL) kindly provided MM.1S (wild-type p53)14 human MM cell line. RPMI8226 and U266 and N.M.); and the Myeloma Research Fund (K.C.A.).

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human MM cells were obtained from American Type Culture Collection (Rockville, MD). All MM cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS; Sigma Chemical, St Louis, MO), 2 μM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Grand Island, NY). Patient MM cells were purified from patient BM aspirates by negative selection using anti-CD2, -CD14, -CD33, -CD41, -CD45RA, and -CD66b antibodies (RosetteSep separation system; StemCell Technologies, Vancouver, BC, Canada). The purity of MM cells is greater than 95%, as confirmed by flow cytometric analysis using anti-CD138 antibody (BD Pharmingen, San Diego, CA).

Reagents
Proteasome inhibitor PS-341 (Millennium Pharmaceuticals, Cambridge, MA) was dissolved in dimethyl sulfoxide (DMSO), stored at -20°C, and used at 5 to 80 nM in the presence of 5% FBS. JNK inhibitor 600215 (Calbiochem, San Diego, CA) was stored at -20°C and used at 5 to 40 μM. Pan-caspase inhibitor Z-AD-FMK and caspase-8 inhibitor Z-IETD-FMK (Calbiochem) were also stored at -20°C and used at 100 μM and 25 μM, respectively. For inhibition of caspase cleavage, cells were incubated with caspase inhibitors for 1 hour before incubation with PS-341 (20 nM) for 8 hours. Caffeine (Sigma) (0.5 and 1 mM) was used as an inhibitor of ATM/ATR.

Growth inhibition assay
MM.1S cells were cultured for 24 hours with 6-25 nM PS-341 in the presence (5-10 μM) or absence of SP600125. The inhibitory effect of PS-341 on MM cell growth in the presence or absence of SP600125 was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Chemicon International, Temecula, CA) dye absorbance, as previously described.14,17

Preparation of biotinylated probes and hybridization on microarrays
MM.1S cells were incubated with 20 nM PS-341 for 0.5, 1, 3, and 6 hours in the presence of 5% FBS. mRNA expression profiling was analyzed using Affymetrix huGene FL arrays (Santa Clara, CA) containing 12,626 genes, as previously described.18

Immunoblotting
MM cell lines and patient MM cells cultured with PS-341 were lysed, and protein lysates (50 μg) were subjected to Western blotting, as in prior studies.11,17 Nuclear extracts were prepared using the Nuclear Extract Kit (Active motif, Carlsbad, CA), according to the manufacturer’s instructions. Antibodies used for immunoblotting included anti-phospho-p53 (Ser6, Ser9, Ser15, Ser20, Ser37, Ser46, Ser392), p53, phospho-SEK1, phospho-SAPK, phospho-c-Jun, phospho-ATF, phospho-Bcl-2, and caspase-8 (Cell Signaling, Beverly, MA); anti-MDM2, -JNK1, -DNA-PKcs, -Bcl-2, -Bcl-XL, -α-tubulin, and -nucleolin (Santa Cruz Biotechnology, Santa Cruz, CA); anti-ATM (Oncogene Research Products, Boston, MA); and anti-caspase-3 (BD Pharmigen). For immunoprecipitation, whole-cell lysates were incubated with anti-p53 and MDM2 overnight at 4°C and then incubated with protein A/G PLUS-agarose (Santa Cruz Biotechnology) for 4 hours at 4°C as in our prior study.14,19

Statistical analysis
Statistical significance of differences observed in drug-treated versus control cultures was determined using the Wilcoxon signed-rank test. The minimal level of significance was P < .05.

Results

**PS-341 up-regulates expression of p53 and MDM2 proteins and induces phosphorylation (Ser15) of p53**

Because p53 is a substrate of UPP, we first examined the effect of PS-341 on p53 protein expression in MM.1S cells. MM.1S cells were treated with PS-341 (20 nM) for up to 16 hours in the presence of 5% FBS. As expected, p53 protein expression was transiently up-regulated after PS-341 treatment in MM.1S cells, with peak expression at 8 hours (Figure 1A). PS-341 also induces transient p53 phosphorylation on Ser15 without the phosphorylation of Ser6, Ser9, Ser20, Ser37, Ser46, or Ser392 residues (data not shown), in the same pattern as p53 protein expression. MDM2 protein is also transiently up-regulated after PS-341 treatment, with peak protein expression observed at 6 hours. We next examined the dose-dependent effect of PS-341 on phosphorylation and protein expression of these proteins. Phosphorylation (Ser15) and protein expression of p53 are up-regulated in a dose-dependent fashion; in contrast, MDM2 expression was decreased only after high-dose PS-341 treatment (Figure 1B). To assess the effect of PS-341 in patient MM cells, we next purified the MM cells from patient bone marrow aspirates. As can be seen in Figure 1C, PS-341 treatment (20 nM, 8 hours) induces the phosphorylation of p53 (Ser15) and up-regulates p53 and MDM2 protein expression in patient MM cells. Because DNA damage induces p53 phosphorylation,20,21 these results suggest that PS-341 induces DNA damage in MM cell lines and patient MM cells. We further examined the distribution and interaction of p-p53, p53, and MDM2 proteins. As expected, most p-p53 is present in the nucleus, and PS-341 triggered increased protein expression associated with the nuclear translocation of p-p53. In contrast, cytoplasmic p53 is in its nonphosphorylated form (Figure 1D). We also examined the association of p53 and MDM2 protein using coimmunoprecipitation with anti-p53 and MDM2 antibodies, and we demonstrated that PS-341 induces coimmunoprecipitation of p53 with MDM2 protein in a time-dependent fashion (Figure 1E). PS-341–induced interaction of p53 and MDM2 occurs earlier than the induction of p53 protein.

**Figure 1. Induction of phosphorylation and protein expression of p53 and MDM2 by PS-341.** (A) PS-341 induces p53 phosphorylation (Ser15) and increased protein expression of p53 and MDM2 in MM.1S cells in a time-dependent fashion. Anti-α-tubulin antibody is used to confirm equal loading of proteins. (B) PS-341 induces p53 phosphorylation (Ser15) and increased protein expression of p53 and MDM2 in MM.1S cells in a dose-dependent fashion. Anti-α-tubulin antibody is used to confirm equal loading of proteins. (C) PS-341 induces p53 phosphorylation (Ser15) and increased protein expression of p53 and MDM2, in purified patient MM cells. Pat indicates patient. (D) Localization of p-p53, p53, and MDM2 proteins in cytoplasmic and nuclear fractions of MM.1S cells treated with PS-341. Anti-α-tubulin and anti-nucleolin antibodies are used to confirm equal loading of cytoplasmic and nuclear proteins, respectively. (E) Coimmunoprecipitation of p53 and MDM2 in MM.1S cells treated with PS-341.
PS-341 induces c-Jun NH₂-terminal kinase activation

We next examined whether PS-341 induces a stress response in MM.1S cells. PS-341 induces the phosphorylation of JNK, and upstream (SEK-1) and downstream (c-Jun and ATF-2) proteins, in a time- (Figure 2A) and dose- (Figure 2B) dependent fashion. PS-341 similarly induces the phosphorylation of JNK in U266 and RPMI 8226 MM cell lines (Figure 2C). As we have done in dexamethasone-treated MM.1S cells, we next performed gene microarray analysis to see if stress response in MM.1S cells before and after PS-341 treatment. As seen in Figure 2D, PS-341 induces a significant (2.5- to 72-fold) increase in heat shock protein (HSP) transcription in a time-dependent fashion. These results indicate that PS-341 induces a stress response in MM cell lines.

Inhibition of JNK activity reduces PS-341–induced cell death by inhibition of caspase-3 activation

To examine whether the activation of JNK plays a role in mediating PS-341–induced apoptosis, we used JNK-specific inhibitor SP600125. In the presence of SP600125, the phosphorylation of JNK triggered by PS-341 is completely abrogated in a dose-dependent fashion (Figure 3A). Phosphorylation of c-Jun or ATF-2 is also blocked by SP600125 (data not shown). SP600125 does not block the phosphorylation of p53 or the induction of MDM2 by PS-341. We further examined whether the inhibition of JNK activity also inhibits caspase-3 cleavage. As seen in Figure 3B, SP600125 inhibits PS-341–induced caspase-3 cleavage, consistent with its inhibition of JNK phosphorylation. SP600125 in a dose-dependent fashion also significantly (∗P < .01) inhibits PS-341–induced cytotoxicity in MM.1S cells, assessed by MTT assay (Figure 3C). These results suggest that JNK plays a critical role in mediating PS-341–induced apoptosis through the activation of caspase-3.

PS-341 induces DNA-PKcs and ATM cleavage in MM

Others have demonstrated that DNA-PKcs is a target for IL-1–converting enzyme (ICE)–like22 and CPP32-like apoptotic protease.23 We have previously reported that PS-341 induces caspase-3 cleavage in MM cells, so we next examined whether DNA-PKcs, ATM, or both are cleaved by caspase activation triggered by PS-341. As expected, PS-341 induces cleavage of DNA-PKcs (180-kDa protein) in a time- (Figure 4A, upper panel), and dose- (Figure 4A, lower panel) dependent fashion in MM.1S cells. PS-341–induced DNA-PKcs cleavage also occurs in RPMI 8226 (Figure 4B, upper panel) and U266 (Figure 4B, lower panel) MM cell lines. Although a constitutive DNA-PKcs cleaved form is present in patient MM cells, PS-341 also induces DNA-PKcs cleavage in these cells (Figure 4C). PS-341 similarly cleaves ATM in a time- (Figure 4D, upper panel), and dose- (Figure 4D, lower panel) dependent fashion in MM.1S cells. These results suggest that PS-341 may inhibit DNA repair by cleavage of DNA-PKcs, ATM, or both in MM cell lines and patient MM cells.

Mechanism by which PS-341 cleaves DNA-PKcs and ATM

Having shown that PS-341 induces DNA-PKcs and ATM cleavage, we next examined the mechanism whereby PS-341 triggers the cleavage of these protein kinases. As in our prior study, PS-341 induces caspase-3 cleavage (Figure 5A, upper panel) and caspase-8 cleavage (Figure 5A, lower panel) in a time-dependent fashion. In contrast, there are no caspase-9 cleavage (data not shown) and no changes in phosphorylation of Bcl-2 or in protein expression of

![Figure 2: PS-341 induces JNK activation and HSP gene expression.](image2)

![Figure 3: JNK inhibitor blocks caspase-3 cleavage and abrogates PS-341–induced apoptosis in MM.1S cells.](image3)

![Figure 4: Cleavage of DNA-PKcs and ATM induced by PS-341.](image4)
Bcl-2 and Bcl-XL (Figure 5B). To determine whether caspase-3 activation mediates DNA-PKcs or ATM cleavage induced by PS-341, we examined the effect of caspase inhibitors on DNA-PKcs and ATM cleavage (Figure 5C). As expected, pan-caspase inhibitor Z-VAD-FMK (4th lane) and caspase-8 inhibitor Z-IETD-FMK (3rd lane) completely abrogate DNA-PKcs and ATM cleavage induced by PS-341. These caspase inhibitors also abrogate PS-341–induced phosphorylation, but not protein expression, of p53 (Figure 5C). Because MDM2 is cleaved during apoptosis,28 we next examined whether PS-341 also cleaves MDM2. As seen in Figure 5D, MDM2 is completely cleaved after treatment with PS-341 for 10 hours. To define the role of ATM or ATR in the phosphorylation of p53 induced by PS-341 treatment, we used caffeine as an inhibitor of ATM23 and ATR.28 Caffeine inhibits PS-341–induced p53 phosphorylation, but not p53 protein expression, in MM.1S cells in a time- (Figure 5E, upper panel) and dose- (Figure 5E, lower panel) dependent fashion. These results suggest that PS-341 induces DNA-PKcs and ATM cleavage, phosphorylation of p53 and MDM2 cleavage through activation of caspase-3 by caspase-8.

**Discussion**

UPP is a major proteolytic system that selectively removes mutant, damaged, or misfolded proteins. It also regulates the expression of proteins mediating cell-cycle progression (p21WAF1, p27KIP1, cyclins), oncogenesis (p53, IκB), and apoptosis (Bcl, cIAP, XIAP, Bax).1 Proteasome inhibitors block UPP and induce caspase activation despite the expression of antipapoptotic proteins (Bcl, cIAP, XIAP) and independent of p53 status. The proteasome inhibitor PS-341 demonstrates remarkable anti-MM activity in vitro11 and in murine MM models,27 and most recently it has achieved stabilization or decrease in paraprotein in 77% of patients with refractory relapsed MM.13 The in vivo targets whereby PS-341 mediates anti-MM activity and, conversely, mechanisms of PS-341 resistance are at present undefined. In this study we define the molecular mechanisms of anti-MM activity of this agent to provide the framework for its optimal clinical use and to form the basis for the development of second-generation, more targeted, potent, and less toxic proteasome inhibitors.

Our previous study demonstrates that PS-341 induces apoptosis in MM cells with wild-type and mutant p53,4 consistent with previous reports that proteasome inhibitor–induced apoptosis occurs dependently3 or independently29 of p53 status. In this study, we hypothesized that DNA damage triggered by PS-341 treatment in MM cells is associated with the activation of DNA-PKcs, ATM/ATR, or both, and the activation of p53. We first report that PS-341 specifically induces the phosphorylation of p53 (Ser15). This induction of p53 phosphorylation is associated with increased p53 protein expression, as previously reported.29 PS-341 induces MDM2 protein and the association of p53 and MDM2 earlier than the phosphorylation of p53. Previous studies have demonstrated that the phosphorylation of p53 (Ser15) dissociates p53 from the p53/MDM2 complex; however, our results demonstrate p53 is still associated with MDM2 even after phosphorylation. Previous reports demonstrated that DNA damage induced the phosphorylation of p53 through the activation of DNA-PKcs;20 therefore, our results strongly suggest that PS-341 induces DNA damage, activates DNA-PKcs, ATM/ATR, or both, and phosphorylates p53 (Ser15) in MM cell lines and primary patient MM cells.

We have studied mechanisms whereby conventional and novel therapies trigger MM cell apoptosis. For example, Dex triggers caspase-9–mediated MM cell death,19 whereas immunomodulatory derivatives of thalidomide and TRAIL induce caspase 8–mediated apoptosis. Our recent gene microarray data of MM.1S cells treated with PS-341 demonstrates transcriptional triggering of apoptotic cascades, down-regulation of growth/survival kinases, up-regulation of UPP, and induction of stress kinases, including heat shock proteins (HSPs). JNK, one of these stress-response
proteins, mediates apoptosis triggered by unfolded proteins.\textsuperscript{33} JNK inhibitor SP600125 blocks PS-341–induced cell death by the abrogation of caspase-3 cleavage but does not affect the phosphorylation of p53. These data suggest that the activation of JNK modulates PS-341–induced caspase activation and apoptosis. Previous reports demonstrate that JNK increases the phosphorylation of antiapoptotic proteins Bcl-2 and Bcl-x, thereby reducing their antiapoptotic activity\textsuperscript{14,33}; however, our data demonstrate that PS-341 does not alter the protein expression of Bcl-2 and Bcl-XL or the phosphorylation of Bcl-2. This result is consistent with our data that PS-341 does not induce the activation of caspase-9 but rather induces caspase-8 apoptotic signaling.

DNA-PKcs is a phosphatidylinositol (PI) kinase that has a crucial role in the repair of damaged DNA and the phosphorylation of selective serine residues (Ser15) on p53,\textsuperscript{20,30} and it is a possible target for an ICE-like protease\textsuperscript{22} or a CPP32-like apoptotic protease.\textsuperscript{23} Therefore, we sought to determine whether the activation of caspases by PS-341 induces cleavage of DNA-PKcs, ATM, or both. We demonstrated that PS-341 cleaves DNA-PKcs and ATM in MM cell lines and primary patient MM cells. PS-341 activates caspase-3 through caspase-8 activation, whereas pan-caspase and caspase-8 inhibitors completely abrogate PS-341–induced caspase-8/caspase-3 activation and DNA-PKcs and ATM cleavage. These data suggest that the cleavage of DNA-PKcs and ATM triggered by PS-341 is dependent on caspase-8/caspase-3 signaling. Our results further demonstrate that the inhibition of caspase activation also inhibits p53 phosphorylation, but not protein expression of p53, suggesting that the phosphorylation of p53 is a secondary event following DNA damage induced by caspase-3. Taken together, our findings indicate that PS-341 activates caspase-3 through caspase-8 activation, impairs DNA repair by the cleavage of DNA-PKcs or ATM, and activates p53 through the phosphorylation of p53 and the degradation of MDM2 (Figure 6). These effects all occur with PS-341 (20 nM) serum levels that are achieved in clinical trials; moreover, PS-341 irreversibly binds to proteasomes and accumulates in target cells. Further delineation of the molecular mechanisms mediating antitumor activity of these agents will provide the framework for their use, alone or coupled with other novel agents, to improve outcome. These studies will also establish the molecular basis for the development of more targeted, potent, and less toxic second-generation proteasome inhibitors.

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