Mechanisms of Proteasome Inhibitor PS-341-induced G_2-M-Phase Arrest and Apoptosis in Human Non-Small Cell Lung Cancer Cell Lines[1]

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ABSTRACT

Purpose: **PS-341 is a novel dipeptide boronic acid proteasome inhibitor with *in vitro* and *in vivo* antitumor activity that induces mechanisms of apoptosis by unknown mechanisms.**

Experimental Design: **Human non-small cell lung cancer cell lines were used to investigate effects PS-341 on cell proliferation, cell cycle progression, and the induction of apoptosis.**

Results: **PS-341 was 38–360-fold more cytotoxic against H460 cells when compared with the proteasome inhibitors MG-132 and PSI. Differential PS-341 cytotoxic effects were found with respect to P53 function: H322 cells (p53 mutant) were 6-fold less sensitive as compared with H460 cells (p53 wild type); and H358 cells (p53 null) were 1.6-fold more sensitive as compared with H460 cells (p53 wild type). A concentration- and time-dependent cell cycle blockade at G_2-M phase was seen for H460 cells without any direct effects on microtubule polymerization or depolymerization. PS-341 exposure in H460 cells led to stabilization of p53, induction of $p21^{cip/waf-1}$ and MDM2 expression, an increase in cyclin B and cyclin A, and the activation of cyclin B and cyclin A kinases. MDM2 induction was found only in H460 cells, whereas in H322 and H358 cells, G_2-M-phase arrest, $p21^{cip/waf-1}$ induction, and an increase in cyclin B1 were found. The commitment of G_2-M-phase cells to apoptosis was verified by the activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase in drug-free medium.**

Conclusions: **Our data suggest that the PS-341-induced G_2-M-phase arrest may be associated with the inhibition of degradation of cell cycle regulators and that the up-regulation of $p21^{cip/waf-1}$ expression may be via p53-dependent and/or -independent pathways. The resulting disturbance of cell cycle progression leads either to growth inhibition or to the initiation of apoptotic pathways.**

INTRODUCTION

The ubiquitin-proteasome pathway plays an important role in the appropriate elimination of intracellular damaged and misfolded proteins and in the rapid proteolysis of a variety of short-lived functional proteins (1–3). A number of proteins, including cell cycle regulators, gene transcription factors, and oncogene proteins, have been identified as substrates for the ubiquitin/proteasome system (4–6). In addition, this system is implicated in the regulation of cell proliferation, differentiation, survival, and apoptosis (7).

Based on the unique potential for cellular regulation via the ubiquitin-proteasome pathway, a number of proteasome inhibitors have been developed and shown to be potently cytotoxic against a variety of cancer cell lines *in vitro* and *in vivo* (8). PS-341 is a novel dipeptide boronate proteasome inhibitor developed by Millennium Pharmaceuticals Inc. (Cambridge, MA) that has been shown to induce apoptosis in prostate cancer cells by blocking cell cycle at G_2-M phase along with the induction of p21 protein accumulation (9, 10). Although PS-341 displays marked anticancer activity in both *in vitro* and *in vivo* systems, the mechanisms of antitumor action need to be further elucidated. Using human non-small cell lung cancer cell lines, we examined the effect of PS-341 on cell proliferation, cell cycle progression, stabilization of p53 protein, induction of p53-inducible gene products, and the triggering of apoptotic pathways. We examined whether the PS-341-induced G_2-M-phase arrest could be related to disruption of microtubule assembly and disassembly. We also explored whether the effects on G_2-M phase are dependent on p53 function, and whether blockage of G_2-M phase is associated with the initiation of apoptotic cascades such as the activation of caspase-3 and PARP[3] protein cleavage. Our results demonstrate that PS-341 treatment causes cells to remain in G_2-M-phase arrest and leads to an increase in accumulation of p53, $p21^{cip/waf-1}$, and MDM2 as well as cyclin A and cyclin B in H460 cells. Although the stabilization of p53 protein was found only in p53 wt cells, the G_2-M-phase arrest

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[3] The abbreviations used are: PARP, poly(ADP-ribose) polymerase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TBST, Tris-buffered saline with Tween 20; wt, wild-type; mt, mutant; NSCLC, non-small cell lung cancer.

and induction of $p21^{cip/waf-1}$ protein were only partly dependent on cellular p53 function. These findings provide insights into the unique mechanisms of antitumor effects of PS-341 and may be used to seek surrogate end points of drug actions in clinical studies.

MATERIALS AND METHODS

Chemicals. PS-341 was supplied by Millennium Pharmaceuticals Inc. and dissolved in DMSO (10 mM) as a stock solution and diluted to the desired concentration with PBS. MG-132 (Z-LLL-CHO) and PSI [Z-IE(OtBu)AL-CHO] were purchased from Bachem Bioscience Inc. (King of Prussia, PA). Monoclonal antibodies were obtained from Calbiochem (Cambridge, MA), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and PharMingen, Inc. (San Diego, CA), respectively. Other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Cell Culture and Cytotoxicity Assays. H460 (p53 wt), H358 (p53-null), and H322 (p53 mt) cell lines were obtained from American Type Culture Collection (Manassas, VA). All cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air.

H460 cells were exposed to varying concentrations of PS-341, MG-132, and PSI for 72 h, and then the cytotoxicity was determined by a MTT assay. H460 (p53 wt) cells, H358 (p53-null) cells, and H322 (p53 mt) cells were chosen for evaluation of the cytotoxic properties of PS-341 as a function of p53 status. Log-phase growing cells were continuously exposed to varying concentrations of PS-341 for 72 h, and drug-induced cytotoxicity was assessed by a MTT assay, as described previously (11).

Cell Cycle Analysis. Cells were treated with various concentrations of PS-341 for 12 h or with 0.1 μM PS-341 for the indicated times. Cells were harvested, fixed with 75% ethanol at −20°C overnight, and then incubated at room temperature for 3 h with 5 μg/ml propidium iodide and 5 μg/ml RNase I (Roche Molecular Biochemicals, Indianapolis, IN). The numbers of cells at different cell cycles and the apoptotic cells (sub-G_1) were measured by flow cytometry (Epics Profile Analyzer; Coulter Co., Miami, FL). Mitotic cells were manually counted on a Nikon microscope after cells were stained with Wright-Giemsa dye solution.

Determination of Microtubule Assembly-Disassembly. The assessment of microtubule assembly-disassembly was performed as described previously (12). Briefly, the inhibition of microtubule assembly was determined in a reaction solution containing 100 μl of β-tubulin solution (500–600 μg protein/ml), 0.1 M 4-morpholinepropanesulfonic acid, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA, and 2.5 M glycerol. After the addition of either 2 μM PS-341, 23 μM paclitaxel, or 22 μM vinblastine, 1 mM GTP was added to the reaction system, and the microtubule assembly process was followed by absorbance changes at 350 nm at room temperature every 5 min, using a Ultrospec III spectrophotometer (Pharmacia LKB, Uppsala, Sweden). The monitoring of the tubulin polymerization required 30 min. For assay of inhibition of microtubule disassembly, compounds at the indicated concentration were added to a prepolymerized microtubule system as described above and incubated in an ice bath. Changes of absorbance were monitored at 350 nm for 20 min, until the absorbance values in the control returned to the starting level, *i.e.*, the completion of assembly-disassembly cycle. DMSO added as a control had no effect on either the assembly or disassembly process.

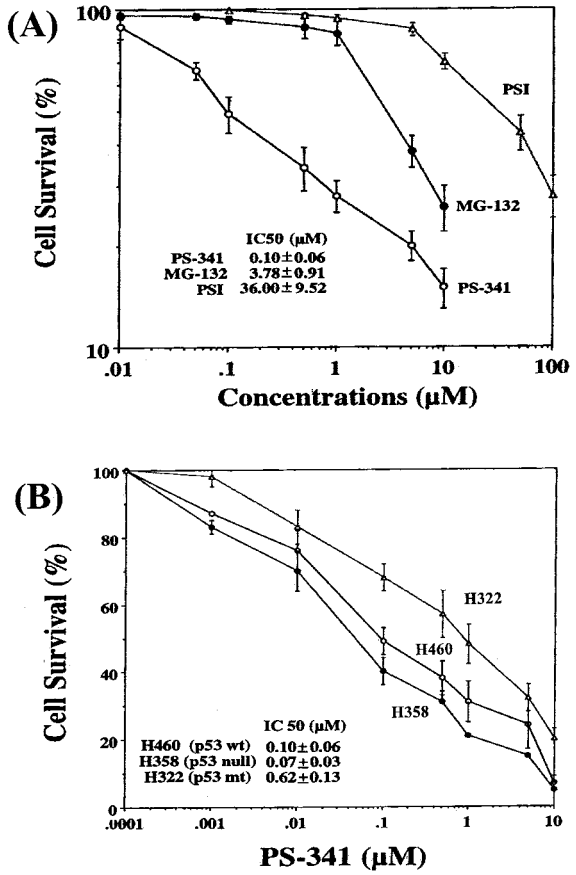

Fig. 1 Effects of proteasome inhibitors on cell survival in human NSCLC cell lines. *A*, H460 cells were exposed to various concentrations of proteasome inhibitors (PS-341, MG-132, and PSI) for 72 h. *B*, H460, H358, and H322 cells were exposed to various concentrations of PS-341 for 72 h. After exposure, cell survival was determined by a MTT assay, and the IC_{50} was calculated graphically as described in "Materials and Methods." Each *point* represents the mean ± SD of three independent experiments.

Western Blot Analysis. Cells were scraped from the culture, washed twice with PBS, and then suspended in 30 μl of Western blot lysis buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 0.1% Triton X-100, and 1% SDS at 0–4°C for 15 min. After centrifugation at 1500 × *g* for 10 min at 0°C, the supernatants were collected, and the proteins were separated on either 12% or 15% SDS-PAGE. After electrophoresis, protein blots were transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in TBST and incubated overnight with the corresponding primary antibodies at 4°C. After washing three times with TBST, the membrane

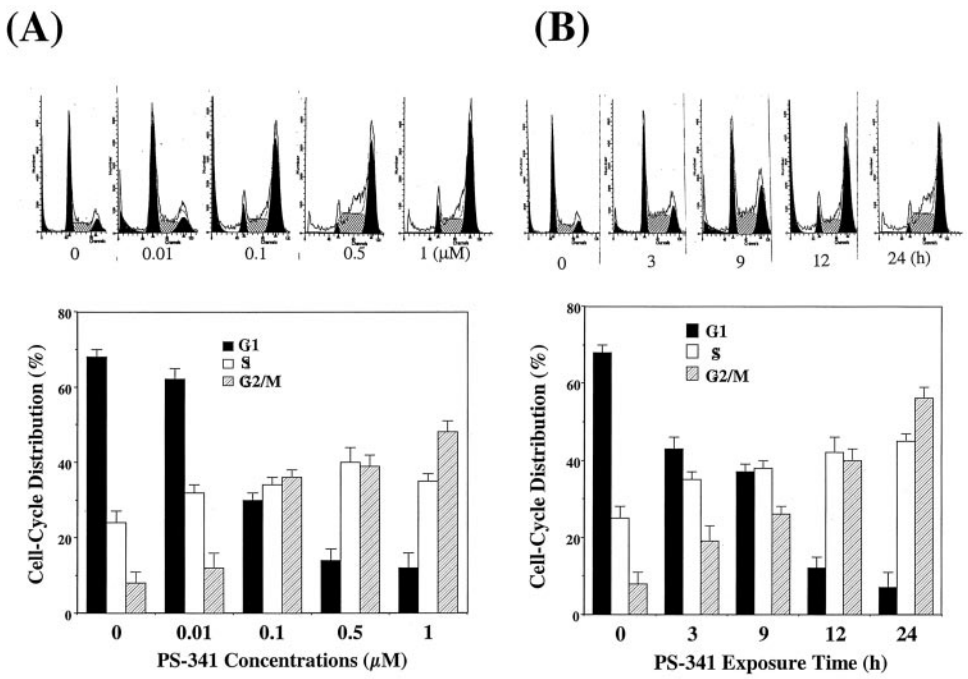

Fig. 2 PS-341 induces G_2-M-phase arrest in H460 cells. H460 cells were exposed to various concentrations of PS-341 for 24 h (*A*) or to 0.1 μM PS-341 for the indicated times (*B*). After exposure, H460 cells were harvested and fixed with cold 75% ethanol overnight. Cells were incubated with 1 μg/ml propidium iodide and 5 μg/ml RNase I at room temperature for 3 h. The profiles of DNA content in each sample (measured by a flow cytometer) are presented in the *top panels*. Each *bar* standing for cell cycle distributions presented in the *bottom panels* represents the mean ± SD of three independent experiments.

was incubated at room temperature for 1 h with horseradish peroxidase-conjugated secondary antibody diluted with TBST (1:1000). The detected protein signals were visualized by an enhanced chemiluminescence reaction system (Amersham, Arlington Heights, IL).

Immunoprecipitation and Kinase Assay. Cells (1×10^6) were treated with lysis buffer containing 0.1% Triton X-100 on an ice bath for 30 min. After centrifugation at 600 × *g* for 5 min, the supernatant fraction was collected and incubated with 10 μg/ml monoclonal anti-cyclin A, anti-cyclin B, anti-cyclin D, and anti-cyclin E antibodies and 10 μl of protein A/G-conjugated agarose (Santa Cruz Biotechnology, Inc.) at 4°C for 2 h. After washing three times with lysis buffer and once with reaction buffer, immunoprecipitants were incubated in 50 μl of reaction mixture containing 30 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 50 μg/ml histone H1, 10 μM ATP, and 20 μCi of [γ-^{32}P]ATP. After incubation at 30°C for 15 min, the reaction was terminated with the addition of 10 μl of 4× Laemmli sample buffer. Histone H1 was separated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. The ^{32}P incorporation into histone H1 was quantified by liquid scintillation counting of the excised gel pieces. The relative kinase activity expressed in treated cells was compared with that in control cells.

Immunocytochemical Studies. Cells were grown on glass coverslips overnight and treated with 0.5 μM paclitaxel, 0.1 μM vinblastine, 0.1 μM PS-341, or the same volume of PBS as control. After treatment, cells were fixed with cold methanol at −20°C for 5 min and then washed three times with 0.1% Tween 20 in PBS and twice with PBS alone. The fixed cells were incubated with monoclonal anti-β-tubulin antibody in 1% BSA/PBS at room temperature for 1 h. After being washed three times with PBS, cells were reincubated with FITC-conjugated secondary antibodies for 30 min in a darkroom. The immunofluorescence complexes were visualized with a Nikon 200 fluorescence microscope.

Assessment of the Ability of PS-341-induced Mitotic Cells to Undergo Apoptosis. H460 cells were treated with 0.1 μM PS-341 for 18 h, and then the mitotic cells were collected by gently shaking. After washing three times with medium, the mitotic cells were reincubated in drug-free fresh medium with 10% fetal bovine serum for 6–48 h. At the end of incubation, cells were collected and divided into three parts. One of cell samples was prepared for determination of mitotic cell numbers after staining cells with Wright-Giemsa dye solution and determination of cell death by trypan blue exclusion. The second part of the cell sample was used for assessments of activity of caspase-3 by measuring the release of pNA from the caspase-3 substrate Ac-DEVD-pNA, and the third part of the cell sample was subjected to analysis of procaspase-3 and PARP cleavage by the Western blot analyses.

RESULTS

PS-341 Inhibits Cell Proliferation in Human NSCLC Cell Lines. The PS-341-induced cytotoxic effects were initially compared with those induced by other proteasome inhibitors, MG-132 and PSI, in H460 cells. PS-341 exhibited a better activity in killing cells compared with the more commonly used proteasome inhibitors, MG-132 and PSI (Fig. 1*A*). The IC_{50} value for PS-341 was 38- and 360-fold lower than that for MG-132 or PSI, respectively. The PS-341-induced cytotoxicity was correlated with p53 function. H322 cells with p53 mt exhibited a 6-fold greater resistance to PS-341 when compared with H460 cells, which were p53 wt (Fig. 1*B*), whereas H358 cells (p53 null) were 1.6-fold more sensitive to PS-341.

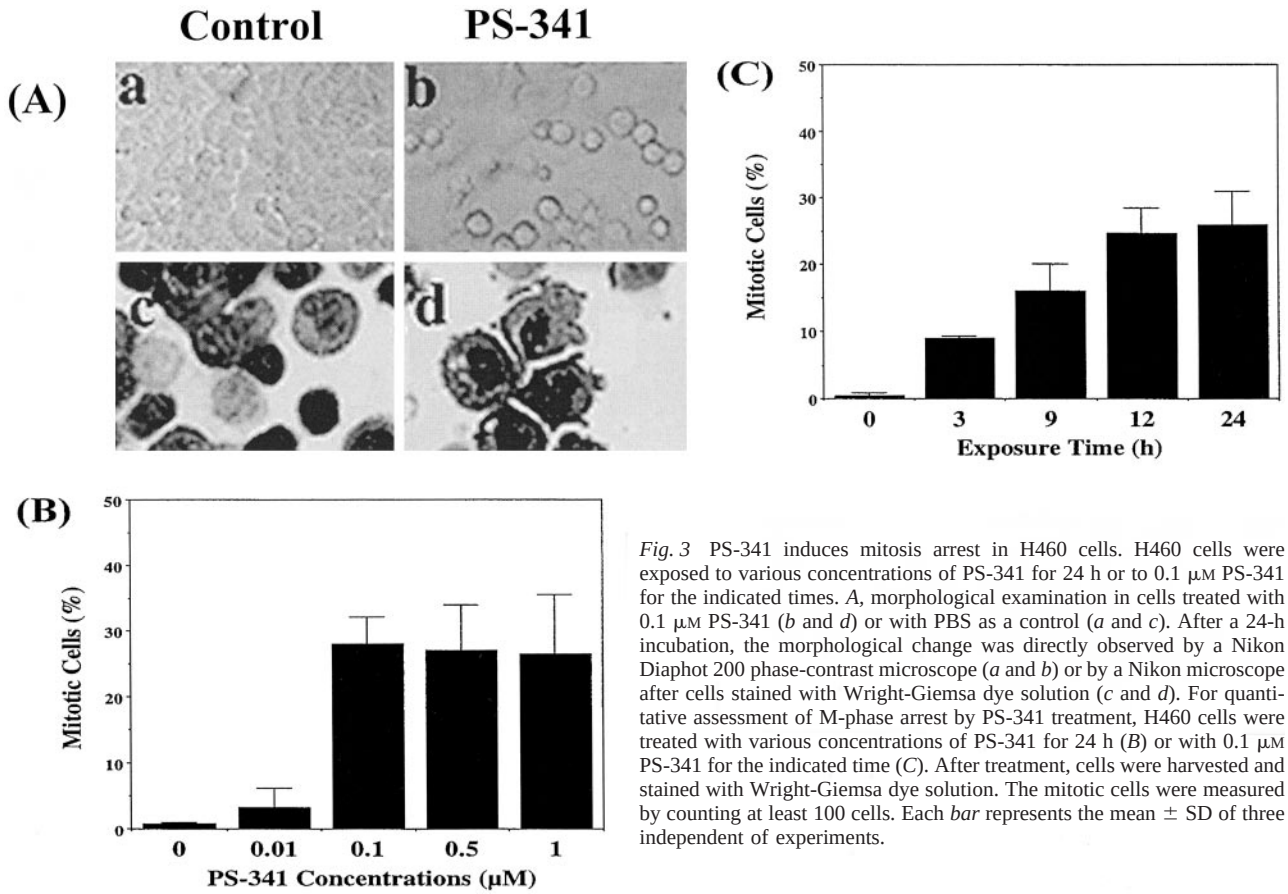

Fig. 3 PS-341 induces mitosis arrest in H460 cells. H460 cells were exposed to various concentrations of PS-341 for 24 h or to 0.1 μM PS-341 for the indicated times. *A*, morphological examination in cells treated with 0.1 μM PS-341 (*b* and *d*) or with PBS as a control (*a* and *c*). After a 24-h incubation, the morphological change was directly observed by a Nikon Diaphot 200 phase-contrast microscope (*a* and *b*) or by a Nikon microscope after cells stained with Wright-Giemsa dye solution (*c* and *d*). For quantitative assessment of M-phase arrest by PS-341 treatment, H460 cells were treated with various concentrations of PS-341 for 24 h (*B*) or with 0.1 μM PS-341 for the indicated time (*C*). After treatment, cells were harvested and stained with Wright-Giemsa dye solution. The mitotic cells were measured by counting at least 100 cells. Each *bar* represents the mean ± SD of three independent of experiments.

PS-341 Induces G_2-M-Phase Arrest. Given the previously reported data that PS-341 causes prostate carcinoma PC-3 cells to accumulate at G_2-M phase (10), we sought to extend these findings to H460 cells. As shown in Fig. 2, PS-341 exposure resulted in an increase in the cell numbers at G_2-M phase and a decrease in the cell numbers at G_1 phase in a concentration- and time-dependent manner. PS-341 exposures from 0.1 to 1 μM resulted in a range of 37–48% of cells that accumulated at G_2-M phase, compared with 8% of cells at G_2-M phase in the untreated cells (Fig. 2*A*). Exposure to 0.1 μM PS-341 caused a progression of H460 cells to arrest at G_2-M phase starting at the 3 h exposure, and the cell numbers at G_2-M phase gradually increased over longer exposure times (Fig. 2*B*). PS-341 treatment also caused a slight increase of cells in S phase. After a comparison of treated and untreated cells stained with Wright-Giemsa dye solution, typical and reproducible morphological observations show that PS-341 exposure was consistent with cell cycle blockade at M phase, with the cells becoming rounded, the chromosomes condensed, and the nuclear envelope disappearing as compared with the intact nuclei in untreated cells (Fig. 3*A*). A quantitative analysis of PS-341-induced M-phase arrest showed that this effect occurred in a concentration- and time-dependent manner. Exposures to PS-341 at concentrations as low as 0.01 μM resulted in ~5% of the cells at M phase, compared with control samples (~0.6% of cells at M phase). The peak value of M-phase arrest was found at 0.1 μM PS-341 (~28% of cells). Treatment with 0.1 μM PS-341 resulted in ~9% of the cells arrested at M phase at 3 h, and this level gradually increased to ~28% between 12 and 24 h (Fig. 3, *B* and *C*).

Effect of PS-341 on Microtubule Assembly-Disassembly. Given the specific arrest of cells at M phase, the morphological changes in PS-341-treated cells appeared similar to those in cells that had been treated with antitubulin agents such as paclitaxel and vinblastine. We examined whether PS-341 could directly affect microtubule assembly or disassembly. The microtubules in the PS-341-exposed H460 cells (Fig. 4*A*, *d*) displayed a normal structure and arrangement comparable with that of control cells (Fig. 4*A*, *a*). The positive controls paclitaxel and vinblastine, respectively, caused microtubule polymerization (Fig. 4*A*, *b*) and microtubule depolymerization (Fig. 4*A*, *c*). The effects of PS-341 on tubulin assembly and disassembly were further examined using purified tubulin in a cell-free system. PS-341 did not affect microtubule assembly and disassembly even at a concentration that was 20 times higher than the IC_{50} in cell culture (Fig. 4, *B* and *C*).

PS-341 Effects on the Accumulation and Activation of G_2-M-related Regulators. Given that the degradation of cyclins is regulated by the ubiquitin-proteasome pathway (13), the effects of PS-341 on intracellular levels of cyclins A, B, D, and

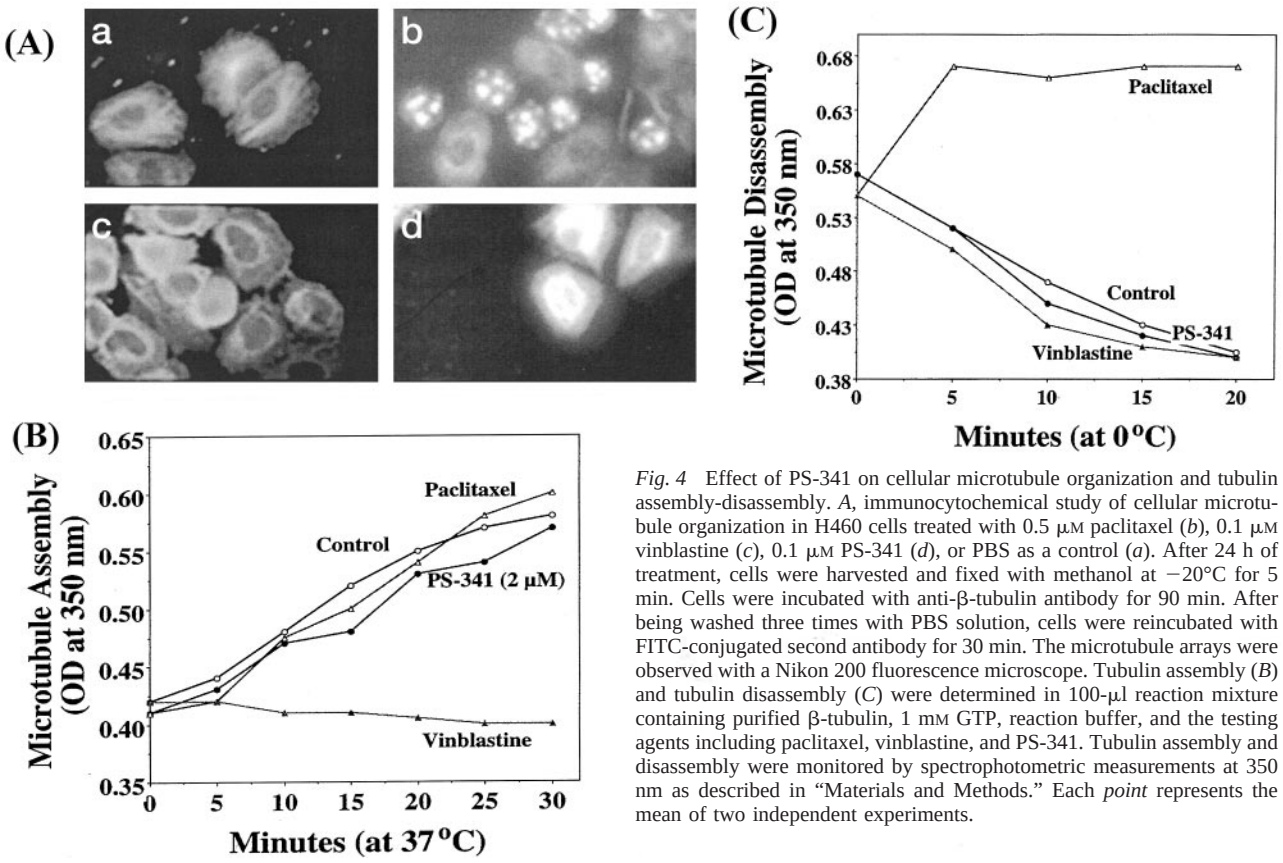

Fig. 4 Effect of PS-341 on cellular microtubule organization and tubulin assembly-disassembly. *A*, immunocytochemical study of cellular microtubule organization in H460 cells treated with 0.5 μM paclitaxel (*b*), 0.1 μM vinblastine (*c*), 0.1 μM PS-341 (*d*), or PBS as a control (*a*). After 24 h of treatment, cells were harvested and fixed with methanol at −20°C for 5 min. Cells were incubated with anti-β-tubulin antibody for 90 min. After being washed three times with PBS solution, cells were reincubated with FITC-conjugated second antibody for 30 min. The microtubule arrays were observed with a Nikon 200 fluorescence microscope. Tubulin assembly (*B*) and tubulin disassembly (*C*) were determined in 100-μl reaction mixture containing purified β-tubulin, 1 mM GTP, reaction buffer, and the testing agents including paclitaxel, vinblastine, and PS-341. Tubulin assembly and disassembly were monitored by spectrophotometric measurements at 350 nm as described in "Materials and Methods." Each *point* represents the mean of two independent experiments.

E and cyclin-dependent kinases were examined. With H460 cells exposed to 0.01–1 μM PS-341 for 24 h or 0.1 μM PS-341 for the indicated times (Fig. 5, *A* and *B*), a marked increase in cyclin B and a slight increase in cyclin A were observed. The levels of cyclin D and cyclin E were not changed along with that of cdc-2 and cyclin-dependent kinase-2. Using histone H1 as a substrate, PS-341 exposure resulted in a marked activation of cyclin B kinase and a slight activation of cyclin A kinase in a time-dependent manner (Fig. 5*C*), but activities of cyclin D and E kinases were not affected (data not shown).

Correlation of p53 Function with PS-341-induced G_2-M-Phase Arrest. Because p53 degradation can be mediated by the ubiquitin-proteasome pathway (14), we examined the effects of PS-341 on p53 and induction of its inducible gene products in H460 cells. PS-341 exposure caused a concentration- and time-dependent accumulation of p53 protein, which was ubiquitinated as demonstrated by the higher molecular weight ladder (Fig. 6, *A* and *B*). The p53-inducible gene products, MDM2 and $p21^{cip/waf-1}$, were found to be markedly induced and showed evidence of accumulation (Fig. 6, *A* and *B*).

Because p53 function is involved in the regulation of cell cycle progression (15), we explored for possible correlations with p53 function and the PS-341-induced cell cycle arrest. Exposure (0.1 μM PS-341) in H460 cells causes a time-dependent blockade of the cell cycle at G_2-M phase (Fig. 7*A*), with the extent of G_2-M-phase arrest paralleling that depicted in Fig. 2*B*. The block of the cell cycle at G_2-M phase was seen in a time-dependent manner for both H358 cells (0.07 μM PS-341), and H322 cells (0.6 μM PS-341). For H460 cells, the extent of PS-341 induced G_2-M-phase blockade was significantly higher than that for H358 and H322 cells: 36–58% of cells were at G_2-M phase at 9 and 24 h after treatment, whereas 22–35% of H358 cells and 26–42% of H322 cells were at G_2-M phase at 9 and 24 h after exposure. The M-phase arrest was assessed by counting metaphase cells detected by staining with Wright-Giemsa. A greater number of H460 cells (~9–28%) were at M-phase after 3–24 h of PS-341 exposure, whereas lesser amounts (~1–6%) of H358 and H322 cells were detected (Fig. 7*B*). Furthermore, a time-dependent increase in p53 protein accumulation with the ubiquitinated higher molecular weight ladder was seen with the PS-341-exposed H460 cells (p53 wt). Non-p53 protein was detected in p53-null H358 cells over these exposure periods. The basal level of p53 protein in H322 cells was markedly higher than that in H460 cells, and p53 level in H322 cells was not changed over the PS-341 exposure time periods. The expression of $p21^{cip/waf-1}$ was markedly induced by PS-341 exposure in a time-dependent manner in all cell lines. The extent of $p21^{cip/waf-1}$ induction in H358 cells was markedly lower than that in H460 and H322 cells. The expression and accumulation of MDM2 protein caused by PS-341 exposure were found only in H460 cells, suggesting that the induction of MDM2 was largely dependent on p53 function. A time-dependent increase in the cyclin B level was seen for all three cell lines used in this work (Fig. 7*B*).

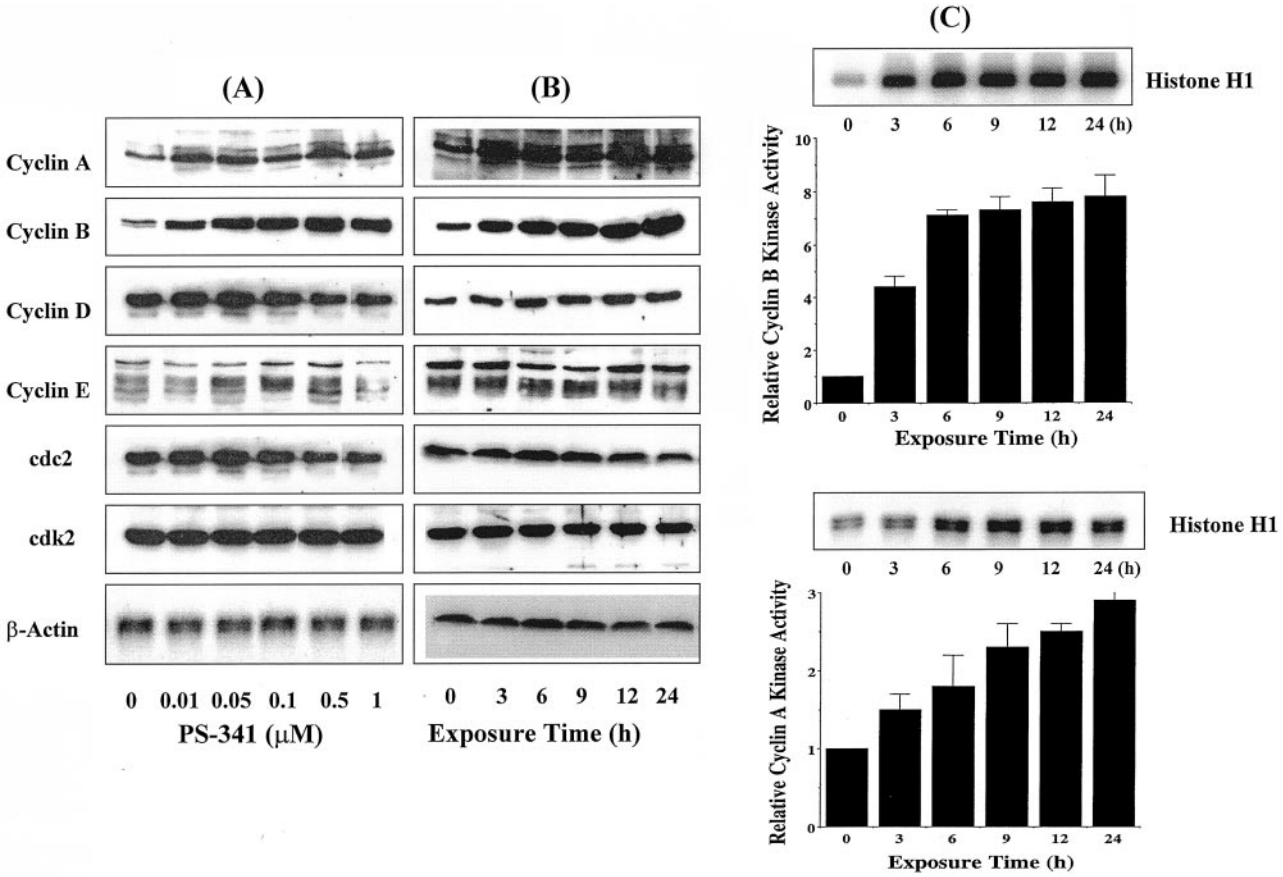

Fig. 5 Effect of PS-341 on the accumulation of cell cycle regulators and the activation of cyclin B1 and cyclin A kinase. H460 cells were exposed to various concentrations of PS-341 for 24 h (*A*) or to 0.1 μM PS-341 for the indicated times (*B*). After exposure, the cells were harvested and prepared for the lysate. Equal amounts (50 μg of protein) of lysates were subjected to 12% SDS-PAGE. After electrotransfer to a membrane, the protein blots were detected by Western blot analysis with corresponding antibodies as described in "Materials and Methods." β-Actin was used as a loading control. *C,* PS-341-induced activation of cyclin B1 and cyclin A kinase. After treatment with 0.1 μM PS-341 for the indicated times, cells were harvested and lysed with lysis buffer. The cyclin B1 and cyclin A were immunoprecipitated by anti-cyclin B1 and anti-cyclin A antibody. The activity of cyclin B1 and cyclin A kinase was determined by [γ-^{32}P]ATP incorporation into histone H1 as described in "Materials and Methods." The relative activity of cyclin B1 and cyclin A was compared with that at time 0. Each *bar* represents the mean ± SD of three independent experiments.

PS-341-induced Mitotic Cells Undergo Apoptosis. PS-341-induced M-phase cells were examined for their ability to reenter cell cycle progression or undergo cell death. To test this possibility, we treated H460 cells with 0.1 μM PS-341 for 18 h, collected drug-induced mitotic cells by gentle shaking, and reincubated the mitotic cells in fresh medium with or without 0.1 μM PS-341 at 37°C for the indicated times. As shown in Fig. 8*A*, the percentage of M-phase cells was ~82% at time 0 and gradually decreased to ~2% after 24–48 h of incubation. In a parallel fashion, the number of dead cells markedly increased proportionally to the increased incubation time (*i.e.*, the percentage of dead cells was ~9% at time 0 and gradually increased to ~58% at 24 h after incubation). The pattern of mitotic cells undergoing cell death in the incubations with PS-341-containing medium was similar to that seen in PS-341-free medium, although more mitotic cells underwent death in the incubations with PS-341-containing medium. To confirm that the cell death was due to apoptosis, the activation of caspase-3 was determined by measurement of the release of pNA from Ac-DEVD-pNA. A 0.5-fold increased caspase-3 activity occurred after a 12-h incubation, and 3.2-fold increased at 24 h postincubation as compared with that at time 0 (Fig. 8*B*). Furthermore, the cleavage of procaspase-3 into activated caspase-3 was seen at 12 h postincubation, and cleavage of PARP protein (an apoptotic hallmark) was observed at 12 h postincubation and increased as a function of incubation time (Fig. 8*C*).

DISCUSSION

PS-341, a boronic acid dipeptide, represents a new generation of proteasome inhibitors (9). *In vitro* studies have shown that boronated proteasome inhibitors are more potent than their structurally analogous aldehydes. PS-341 was 7 times more potent than the general proteasome inhibitor MG-132 in inhibiting purified proteasome activity (16). PS-341 demonstrates potent activity on the growth of human NSCLC cancer cell line H460. Cytotoxicity studies have shown that PS-341 is 38- and 360-fold more potent than MG-132 and PSI, respectively. PS-

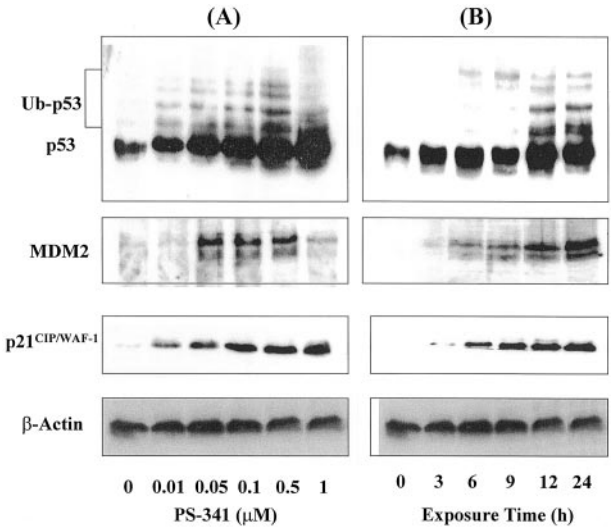

Fig. 6 Effect of PS-341 on the ubiquitination and accumulation of p53, as well as p53-inducible gene products. H460 cells were treated with various concentrations of PS-341 for 24 h (*A*) or with 0.1 μM PS-341 for the indicated times (*B*). After treatment, cells were harvested and prepared for the lysate. Equal amounts (50 μg of protein) of lysates were subjected to 12% SDS-PAGE. After transfer to membrane, the protein blots were detected by Western blot analysis with corresponding antibodies as described in "Materials and Methods." β-Actin was used as a loading control.

341 at low concentrations leads to G_2-M-phase arrest, particularly at M phase in H460 cells. The PS-341 exposure leads to an increase in the accumulation and activation of G_2-M phase-related cycle regulators, cyclin A and cyclin B1, and the activation of cyclin A and cyclin B1 kinases (Fig. 5). In contrast, PS-341 had no effect on the levels of cyclin D and cyclin E and the stimulation of cyclin D and E kinases (data not shown), although degradation of these types of cyclins is also related to the activity of the ubiquitin-proteasome system (17). In addition, PS-341 exposure did not affect the assembly or disassembly of microtubules in a cell-free system, suggesting that blockade of cyclin B degradation and activation of cyclin B kinase may lead to cell cycle blockade at G_2-M phase. Indeed, Sherwood *et al.* (18) have demonstrated that the inhibition of cyclin B degradation and persistence of the activation of cyclin B kinase, caused by the neutral cysteine protease inhibitor, *N*-acetylleucylleucyl-norleucinal, led to the accumulation of Chinese hamster ovary cells at G_2-M phase, particularly at M phase.

It is known that p53 plays important roles in cell cycle regulation and apoptosis (19, 20). Proteasome inhibitor-induced apoptosis has been described as p53 dependent (21); however, another study has shown that the induction of apoptosis by proteasome inhibitors is p53 independent (22). In this work, we used p53 wt H460 cells, p53-null H358 cells, and p53 mt H322 cells as models to correlate the effects of p53 function on PS-341-induced cytotoxicity. Our data demonstrated that p53 mt H322 cells were 6-fold resistant to PS-341-induced cell killing compared with p53 wt H460 cells, suggesting that the PS-341-induced cytotoxicity may be associated with p53 function. However, we also found that p53-null H358 cells were 1.6-fold more sensitive to PS-341 than H460 cells, indicating that the inhibition of cell growth by a proteasome inhibitor such as PS-341 is only partly dependent on cellular p53 function. Also, PS-341 exposure resulted in a time-dependent accumulation of cells at G_2-M phase in all of the tested cell lines, indicating that G_2-M-phase arrest caused by proteasome inhibitor may be via a p53-independent pathway.

For comparable exposures to PS-341, a higher percentage of cells was found at G_2-M phase in H460 cells than in H358 and H322 cells (Fig. 7*A*), suggesting that wt p53 may be associated with enhancement and facilitation of the PS-341 effect on the blockade of the cell cycle at G_2-M phase. In addition, the extent of M-phase arrest by PS-341 in H460 cells was significantly greater than that in H358 and H322 cells (Fig. 7*B*). These findings suggested that the cellular p53 functions may be involved in the proteasome inhibitor-induced M-phase arrest. In H460 cells, PS-341 exposure led to the accumulation of p53 protein with ubiquitinated higher molecular weight ladders that resulted from the inhibition of p53 protein degradation. By contrast, in H322 cells, PS-341 exposure did not cause p53 accumulation, although the basal level of p53 in H322 cells was higher than that in H460 cells (Fig. 7*B*). These data suggested that the mt p53 protein might lose its ability to be a substrate for the ubiquitin-proteasome pathway, leading to a prolonged higher level of p53, which, in turn, caused its dissociation from the regulation of cell proliferation, cell cycle, and apoptosis. These findings are consistent with the observations of Cheng *et al.* (23), who found that effects by proteasome inhibitors increased the expression of at least three p53-responsive gene products (bax, MDM2, and p21) that were independent of cellular p53 function. These investigators felt that it was possible that the p53 response proteins are short-lived and subject to degradation by ubiquitin-proteasome-dependent proteolysis.

It has been known that an increase in p53 results in the induction of expression of several genes (24). For example, the expression of $p21^{cip/waf-1}$, a cyclin/cyclin kinase inhibitor, is regulated by p53 via a transcriptional or posttranscriptional pathway (25). Recent evidence also indicates that the induction of $p21^{cip/waf-1}$ protein may be independent of the p53 pathway (26). We found that exposure to PS-341 dramatically induces $p21^{cip/waf-1}$ protein expression in the p53 wt H460 cells. We also observed that in H358 and H322 cells exposed to equal toxic concentrations of PS-341, a comparable $p21^{cip/waf-1}$ expression was seen (Fig. 7*C*), thus indicating that PS-341-induced $p21^{cip/waf-1}$ expression could occur via a p53-independent pathway. These results are consistent with a report by An *et al.* (27). Although the role of $p21^{cip/waf1}$ in the G_1-S boundary checkpoint has been well documented (28), the role of this protein in the G_2-M checkpoint remains unclear (29). Recent reports describe that a novel gene product, p21CIP1-associated regulator of cyclin B, is predominantly associated with the centrosome and mitotic spindle poles, indicating that $p21^{cip/waf-1}$ may regulate cyclin B1 function and transit through the G_2-M phase of the cell cycle (30). Some reports have shown that $p21^{cip/waf-1}$ plays an important role in the regulation of G_2-M-phase transition through its inhibitory effect on cdc2/cyclin B complex (31). Others show that the disruption of the $p21^{cip/waf-1}$/p53 pathway results in mitotic spindle pole defects and the appearance of multiple centrosomes (32). Moreover,

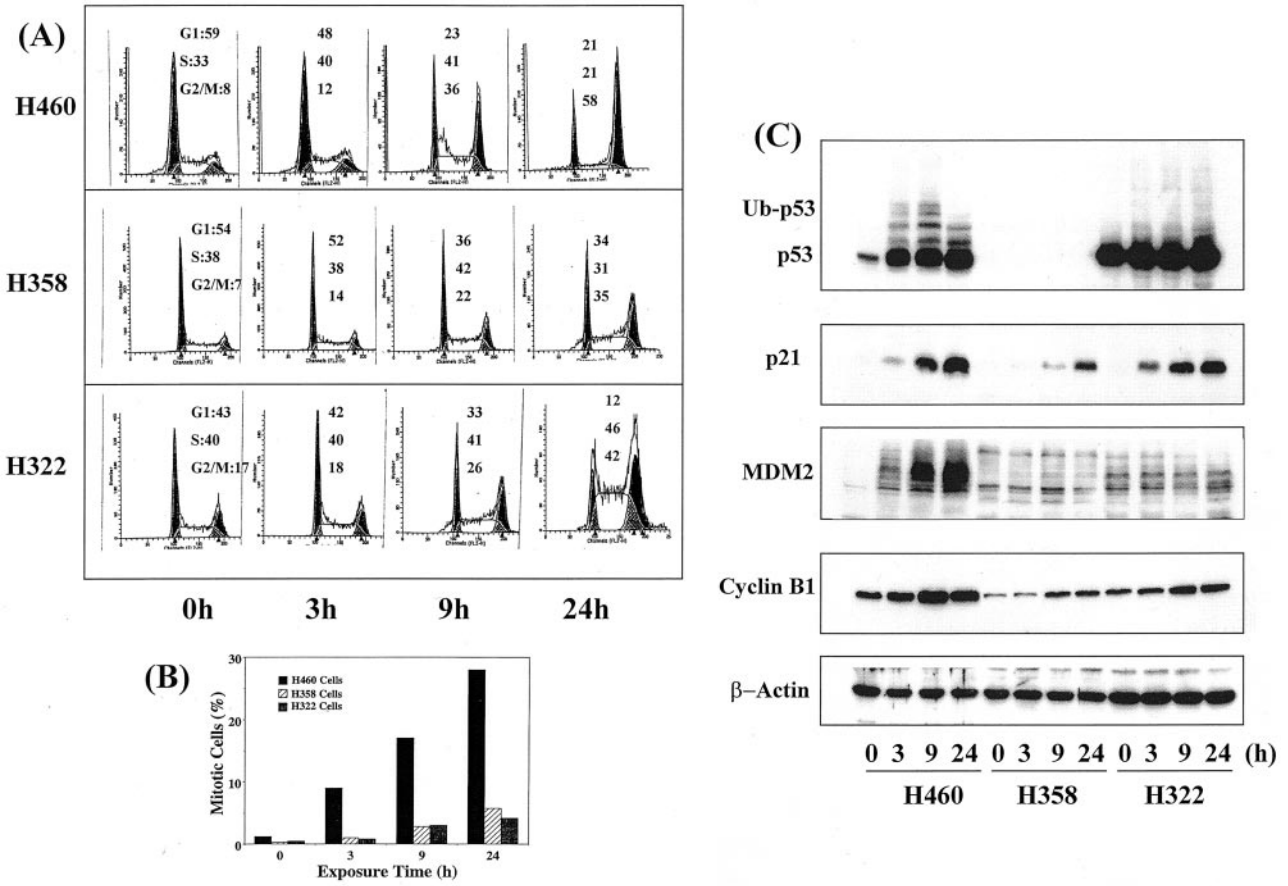

Fig. 7 Effect of cellular p53 function on PS-341-induced G_2-M-phase arrest, p53 stabilization, expression of $p21^{cip/waf-1}$, MDM2, and cyclin B1 in human NSCLC cell lines. *A*, H460, H358, and H322 cells were exposed to an equal toxic concentration of PS-341 for the indicated time. At the time point, cells were harvested, washed with PBS solution, and fixed with cold 75% ethanol overnight. Cells were stained with propidium iodide, and the cellular DNA contents were assessed as described in Fig. 2. *B*, cells were treated with PS-341 as described in *A*. After treatment, cells were harvested and stained with Wright-Giemsa dye solution. The mitotic cells were assessed by using a microscope as described in Fig. 3. *C*, cells were exposed to PS-341 as described in *A*. After exposure, cells were harvested and lysed by lysis buffer. Equal amounts (50 μg of protein) of lysates were subjected to 12% SDS-PAGE. After transfer to a membrane, the protein blots were detected by Western blot analysis with the corresponding antibodies as described in "Materials and Methods." β-Actin was used as a loading control.

Ando *et al.* (33) recently demonstrated that the interaction of $p21^{cip/waf-1}$ with proliferating cell nuclear antigen may contribute to maintenance of the cell cycle blockade at G_2-M phase after DNA damage. Although the $p21^{cip/waf-1}$ protein was markedly induced by PS-341 treatment in all tested cell lines, the G_1-phase blockade was not observed in our experiments. We do not know why an increase in $p21^{cip/waf-1}$ expression by PS-341 did not induce the cells at G_1-phase arrest. One of possibilities could be that some of cellular components that are involved in the G_1-phase arrest, such as Rb, E2F1, and related cyclins, were not activated by PS-341 treatment. The other explanation may be that the mechanisms by which the cell cycle exits G_2-M phase to entering G_1 phase were blocked by PS-341.

PS-341 exposure caused lung cancer cells (H460, H358, and H322) to accumulate at G_2-M phase and elevated the amounts of p53, $p21^{cip/waf-1}$, and cyclin B1. However, we do not know how these proteins interact with each other or which components could be involved in the primary machinery to block the cell cycle at G_2-M phase. MDM2, an oncoprotein, can bind to p53 within transactivation domains and is involved in p53 protein degradation by the ubiquitin-proteasome pathways. Degradation of MDM2 protein is also involved in ubiquitin-proteasome pathways (33). Exposure to PS-341 leads to an increase in MDM2 accumulation in a concentration- and time-dependent manner in p53 wt H460 cells. However, we did not find that PS-341 exposure resulted in the accumulation of this protein in p53-null and p53 mt cells (Fig. 7*C*), suggesting that proteasome inhibitor-induced MDM2 expression might be dependent on p53 pathways. Because MDM2 plays a role in the negative regulation of p53 level and function, the dramatic increase in both p53 and MDM2 proteins in p53 wt cells after treatment with PS-341 could lead to an alteration in the balance between these two proteins, and such a mechanism should be considered as playing a role in triggering of the apoptotic pathways.

It has been proposed that G_2-M-phase arrest caused by DNA-damaging agents or by stress stimuli, could be thought to be provided an opportunity for DNA repair, or in the absence of

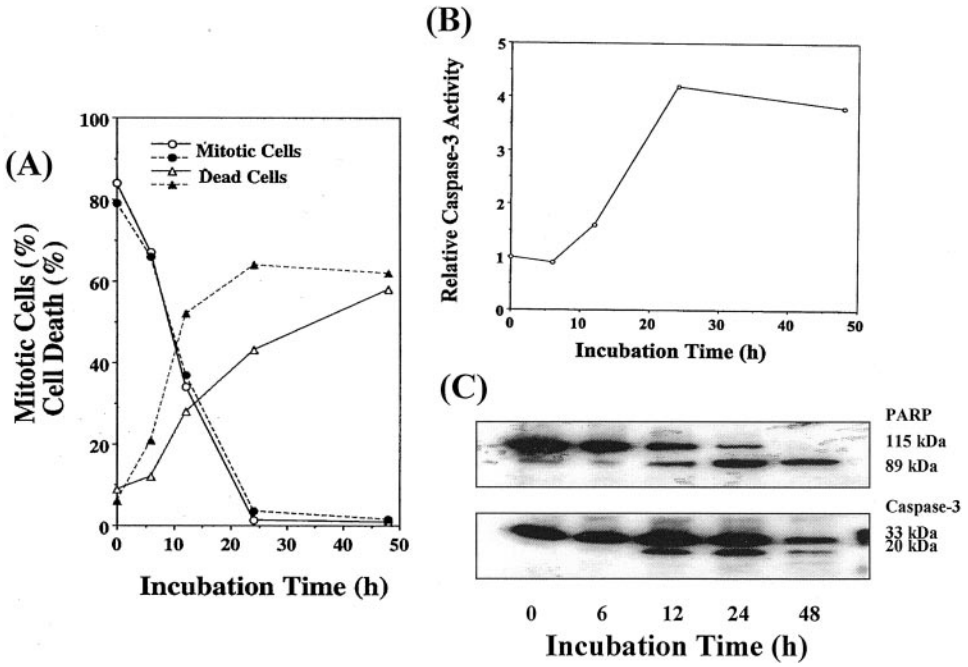

Fig. 8 PS-341-induced mitotic cells undergo apoptotic death. *A,* H460 cells were exposed to 0.1 μM PS-341 for 18 h. After exposure, the mitotic cells were collected by gentle shaking and washed three times with medium. The mitotic cells were reincubated in drug-free fresh medium containing 10% fetal bovine serum at 37°C for the indicated time (*solid line*) or reincubated in the drug-containing medium with 10% fetal bovine serum (*dashed line*). After incubation, cells were taken from culture, and the cell samples were divided into three parts. One part of the sample was subjected to determination of mitotic cell numbers after staining with Wright-Giemsa and determination of dead cell numbers by trypan blue exclusion. *B,* cell sample taken from culture incubated in drug-free medium for the indicated times was prepared for determination of caspase-3 activity by measurement of the release of pNA from Ac-DEVD-pNA as described in "Materials and Methods." *C,* cell sample taken from culture incubated in drug-free medium for the indicated times was subjected to determination of PARP and procaspase-3 cleavage by Western blot analysis.

such a mechanism, the mitotic cells will be premature and result in a lethal outcome. One of the modes for nonapoptotic cell death after G_2-M-phase arrest has been described as "mitotic death" or "mitotic catastrophe" (34, 35). Mitotic death often occurs in G_2-M-phase-arrested cells after incomplete or defective mitosis (36). In this work, we have examined the fate of PS-341-induced G_2-M cells. It was apparent from the increase in the numbers of dead cells and the decreased cell numbers at M phase (Fig. 8*A*), that PS-341-induced mitotic cells, when followed by reincubation in fresh drug-free medium, led to the process of the mitotic cells undergoing cell death. The activity of caspase-3 was activated at 12 h after incubation and markedly increased after 24 h. The cleavage of procaspase-3 and PARP protein also was predominantly detected at 12 h after incubation, and cleavage gradually increased at 24 and 48 h. All these data indicate that G_2-M-phase arrest caused by PS-341 leads to the initiation of apoptotic signaling.

In summary, we have demonstrated that exposure of several NSCLC cell lines to PS-341 at low concentrations results in cell cycle arrest at G_2-M phase and induction of apoptosis. Unlike antitubulin agents, the PS-341-induced G_2-M-phase arrest is not due to a direct effect on microtubule polymerization and depolymerization. It is apparent that much of the activity of PS-341 is due to the proteasome-specific effects of delayed degradation via ubiquitin-dependent proteolysis. This then impacts the cell cycle regulation process, where the inability of the recycling of ubiquinated proteins has direct effects on the normal cell cycle machinery by inducing elevated expression of many proteins that are normally short-lived. Our data have also demonstrated that PS-341 has significant antitumor activity against human NSCLC cell lines, regardless of p53 status, suggesting that PS-341 has a role in the inhibition of tumor growth via both p53-dependent and -independent mechanisms. PS-341 has the potential to be a potent therapeutic agent used alone and in combination with conventional chemotherapeutics for human cancers, regardless of p53 status.

REFERENCES

1. Hochstrasser, M. Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. Curr. Opin. Cell Biol., *7:* 215–223, 1995.

2. Juntsch, S., and Schlenker, S. Selective protein degradation: a journey's end within the proteasome. Cell, *82:* 881–884, 1995.

3. Hershko, A., and Ciechanover, A. The ubiquitin system. Annu. Rev. Biochem., *67:* 425–480, 1998.

4. Desai, S. D., Liu, L. F., Vazquez-Abaad, D., and D'Arpa, P. Ubiquitin-dependent destruction of topoisomerase I is stimulated by the antitumor drug camptothecin. J. Biol. Chem., *272:* 24159–24164, 1997.

5. Ciechanover, A., DiGiuseppe, A. J., Bercovich, B., Orian, A., Richter, J. D., Schwartz, A. L., and Brodeur, G. M. Degradation of nuclear oncoproteins by the ubiquitin system *in vitro*. Proc. Natl. Acad. Sci. USA, *88:* 139–143, 1991.

6. Treier, M., Staszewski, L. M., and Bohmann, D. Ubiquitin-dependent c-jun degradation *in vivo* is mediated by the δ domain. Cell, *78:* 787–798, 1994.

7. Grimm, L. M., Gildberg, A. L., Porier, G. G., Schwartz, L. M., and Osborne, B. A. Proteasomes play an essential role in thymocyte apoptosis. EMBO J., *15:* 3835–3844, 1996.

8. Drexler, H. C., Risau, W., and Konerding, M. A. Inhibition of proteasome function induces programmed cell death in proliferating endothelial cells. FASEB J., *14:* 65–77, 2000.

9. Adams, J., Behnke, M., Chen, S., Cruickdhank, A. A., Dick, L. R., Grenier, L., Klunder, J. M., Ma, Y-T., Plamondon, L., and Stein, R. L. Potent and selective inhibitors of the proteasome: dipepidyl boronic acids. Bioorg. Med. Chem. Lett., *8:* 333–338, 1998.

10. Adams, J., Palombella, V. J., Sausville, E. A., Jonson, J., Destree, A., Lazarus, D. D., Maas, J., Pien, C. S., Prakash, S., and Elliot, P. J. Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res., *59:* 2615–2622, 1999.

11. Ling, Y. H., Priebe, W., Yang, L-Y., Burke, T. G., Pommier, Y., and Perez-Soler, R. *In vitro* cytotoxicity, cellular pharmacology, and DNA lesions induced by annamycin, an anthracycline derivative with high affinity for lipid membranes. Cancer Res., *53:* 1583–1589, 1993.

12. Jiang, J-D., Davis, A. S., Middleton, K., Ling, Y. H., Perez-Soler, R., Holland, J. F., and Bekesi, J. G. 3-(Iodoacetmido)-bezoylurea: a novel cancericidal tubulin ligand that inhibits microtubule polymerization, phosphorylates bcl-2, and induces apoptosis in tumor cells. Cancer Res., *58:* 5389–5395, 1998.

13. Koepp, D. M., Harper, J. W., and Elledge, S. J. How the cyclin became a cyclin: regulated proteolysis in the cycle. Cell, *97:* 431–434, 1999.

14. Oren, M. Regulation of the p53 tumor suppressor protein. J. Biol. Chem., *274:* 36031–36043, 1999.

15. Cox, L. S., and Lane, D. P. Tumour suppressors, kinase and clamps: how p53 regulates the cell cycle. BioEssays, *17:* 501–508, 1995.

16. Grisham, M. B., Palombella, V. J., Elliott, P., Conner, E. M., Brand, S., Wong, H. L., Pien, C., Mazzola, L. M., Destree, A., Parent, L., and Adams, J. Inhibition of NF-κB activation *in vitro* and *in vivo*: role of 26S proteasome. Methods Enzymol., *300:* 345–363, 1999.

17. Glotzer, M., Murray, A. W., and Kirschner, M. W. Cyclin is degradation by the ubiquitin pathway. Nature (Lond.), *349:* 132–138, 1991.

18. Sherwood, S. W., Kung, A. L., Roitelman, J., Simoni, R. D., and Schimke, R. T. *In vivo* inhibition of cyclin B degradation and induction of cell-cycle arrest in mammalian cells by the neutral cysteines protease inhibitor *N*-acetylleucylnorleucinal. Proc. Natl. Acad. Sci. USA, *90:* 3353–3357, 1993.

19. Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V., and Kastan, M. B. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA, *89:* 7491–7495, 1992.

20. Wu, X., and Levine, A. p53 and E2F-1 cooperate to mediate apoptosis. Proc. Natl. Acad. Sci. USA, *91:* 3602–3606, 1994.

21. Lopes, U. G., Erhardt, P., Yao, R., and Cooper, G. M. P53-dependent induction of apoptosis by proteasome inhibitors. J. Biol. Chem., *272:* 12893–12896, 1997.

22. Shinohara, K., Tomioka, M., Nakan, H., Tone, S., Ito, H., and Kawashim, S. Apoptosis induction resulting from proteasome inhibition. Biochem. J., *317:* 385–388, 1996.

23. Cheng, Y. C., Lee, Y. S., Tejima, T., Tanaka, K., Omura, S., Heintz, N. H., Mitsui, Y., and Magae, J. Mdm2 and Bax, downstream mediators of the p53 response, are degraded by the ubiquitin-proteasome pathway. Cell Growth Differ., *9:* 79–84, 1998.

24. Agarwal, M. L., Taylor, W. R., Chernov, M. V., Chernova, O. B., and Stark, G. R. The p53 network. J. Biol. Chem., *273:* 1–4, 1998.

25. Mocleod, K. F., Sherry, N., Hannon, G., Beech, D., Tokino, T., Kinzler, K., Vogelstein, B., and Jacks, T. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. Genes Dev., *9:* 935–944, 1995.

26. Michieli, P., Chedid, M., Lin, D., Peirce, J. H., Mecer, W. E., and Givol, D. Induction of WAF1/CIP1 by a p53-independent pathway. Cancer Res., *54:* 3391–3395, 1994.

27. An, W. G., Hwang, S-G., Trepel, J. B., and Blagosklonny, M. V. Proteasome inhibitor-induced apoptosis; accumulation of wt p53, $p21^{WAF1/CIP1}$, and induction of apoptosis are independent markers of proteasome inhibition. Leukemia (Baltimore), *14:* 1276–1283, 2000.

28. LaBaer, J., Garrett, M. D., Stevenson, L. F., Slingerland, J. M., Sandhu, C., Chou, H. S., Fattaey, A., and Harlow, E. New functional activities for the p21 family of CDK inhibitors. Genes Dev., *11:* 847–862, 1997.

29. Nunez, F., Chipchase, M. D., Clarke, A. R., and Melton, D. W. Nucleotide excision repair gene (ERCC1) deficiency causes G_2 arrest in hepatocytes and a reduction in liver binuclation: the role of p53 and p21. FASEB J., *14:* 1073–1082, 2000.

30. McShea, A., Samuel, T., Eppel, J-T., Galloway, D. A., and Funk, J. O. Identification of CIP-1-assoaciated regulator of cyclin B (CARB), a novel p21-binding protein acting in the G_2 phase of the cell cycle. J. Biol. Chem., *275:* 23181–23186, 2000.

31. Guadagno, T. M., and Newport, J. W. Cdk2 kinase is required for entry into mitosis as a positive regulator of cdc2-cyclin B kinase activity. Cell, *84:* 73–82, 1996.

32. Mantel, C., Braun, S. E., Reid, S., Henegariu, O., Liu, L., Hangoc, G., and Broxmeyer, H. E. $P21^{Cis\text{-}1/Waf\text{-}1}$ deficiency causes deformed nuclear architecture, centriole overduplication, polyploidy, and relaxed microtubule damage checkpoints in human hematopoietic cells. Blood, *93:* 1390–1398, 1999.

33. Ando, T., Kawabe, T., Ohara, H., Ducommun, B., Itoh, M., and Okamoto, T. J. Involvement of the interaction between p21 and proliferating cell nuclear antigen for the maintenance of G_2/M arrest after DNA damage. J. Biol. Chem., *276:* 42971–42977, 2001.

34. King, K. L., and Cidlowski, J. A. Cell cycle and apoptosis: common pathways to life and death. J. Cell. Biochem., *58:* 175–180, 1995.

35. Sleimam, R. J., and Stewart, B. W. Early caspases activation in leukemic cells subject to etoposide-induced G_2-M arrest: evidence of commitment to apoptosis rather than mitotic death. Clin. Cancer Res., *6:* 3756–3765, 2000.

36. Bracey, T. S., Williams, A. C., and Paraskeva, C. Inhibition of radiation-induced G_2 delay potentiates cell death by apoptosis and/or induction of giant cells in colorectal tumor cells with disrupted p53 function. Clin. Cancer Res., *3:* 1371–1378, 1997.

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