Vitamin C Inactivates the Proteasome Inhibitor PS-341 in Human Cancer Cells

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Abstract **Purpose:** PS-341 (bortezomib, Velcade), the first proteasome inhibitor approved by the Food and Drug Administration for the treatment of patients with relapsed multiple myeloma, induces apoptosis in human cancer cell lines. Vitamin C (ascorbic acid) is an essential water-soluble vitamin required for many normal physiologic functions and has to be obtained through diet or supplemental tablets in humans. Here we studied the potential effect of vitamin C on the anti-cancer activity of PS-341 in human cancer cell lines.

Experimental Design: The effects of vitamin C on apoptosis induction by PS-341 alone and by PS-341 combined with tumor necrosis factor – related apoptosis-inducing ligand were studied. In addition, the effects of vitamin C and other antioxidants on PS-341-mediated proteasome inhibition were also examined. Finally, the direct chemical interaction between vitamin C and PS-341 was determined.

Results: Vitamin C abrogated the ability of PS-341 to induce apoptosis in various human cancer cell lines, to induce G_2-M arrest, and to augment apoptosis induced by tumor necrosis factor – related apoptosis-inducing ligand. Moreover, vitamin C suppressed PS-341-mediated inhibition of proteasome activity. PS-341 itself did not induce generation of intracellular reactive oxygen species whereas other antioxidants failed to abrogate its biological activity. Importantly, we detected a direct chemical interaction between vitamin C and PS-341.

Conclusion: Vitamin C directly binds to PS-431, thus inactivating PS-341 independent of its antioxidant activity. Our findings suggest that vitamin C may have a negative effect on PS-341-mediated anticancer activity.

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PS-341 (bortezomib, Velcade; Fig. 1) is a dipeptidyl boronic acid proteasome inhibitor with proven anticancer activity. PS-341 alone or in combination with other cytotoxic or targeted agents induces apoptosis in a variety of human cancer cells *in vitro* and inhibits the growth of tumor xenografts animal models. In addition, PS-341 can function as a chemosensitizer or radiosensitizer to overcome tumor resistance to drugs or radiotherapy. Therefore, PS-341 was the first proteasome inhibitor tested in clinical trials and was recently approved by the Food and Drug Administration for the treatment of patients with relapsed multiple myeloma (1). Currently, clinical trials that are testing the anticancer efficacy of PS-341 or its combinations with other agents in a number of hematologic and solid tumors are ongoing.

Vitamin C (also called ascorbic acid; Fig. 1) is an essential water-soluble vitamin present in plant and animal cells. Humans cannot synthesize vitamin C *de novo* and thus have to acquire most body storage of vitamin C through fruits and vegetables or vitamin supplements. The average healthy adult has a body pool of 1.2 to 2.0 g of vitamin C that may be maintained with at least 75 mg/d of vitamin C intake (2).

Vitamin C was reported to increase the survival of cancer patients albeit with controversy (3 – 5). Epidemiologic evidence points to the capacity of vitamin C to prevent cancer at a number of sites (3). Preclinical studies have shown that vitamin C alone or together with other antioxidants potentiates the efficacy of other cancer therapeutic agents (6 – 13). One clinical case report showed that vitamin C together with other oxidants, when added adjunctively to first-line chemotherapy, prevented recurrence in two ovarian cancer patients (14), suggesting that vitamin C and other antioxidants may improve the efficacy of chemotherapy (15).

In this study, we studied the effects of vitamin C and other antioxidants on PS-341-induced apoptotic cell death in human cancer cells. Interestingly, vitamin C was the only one that inhibited PS-341-induced apoptotic death among the tested

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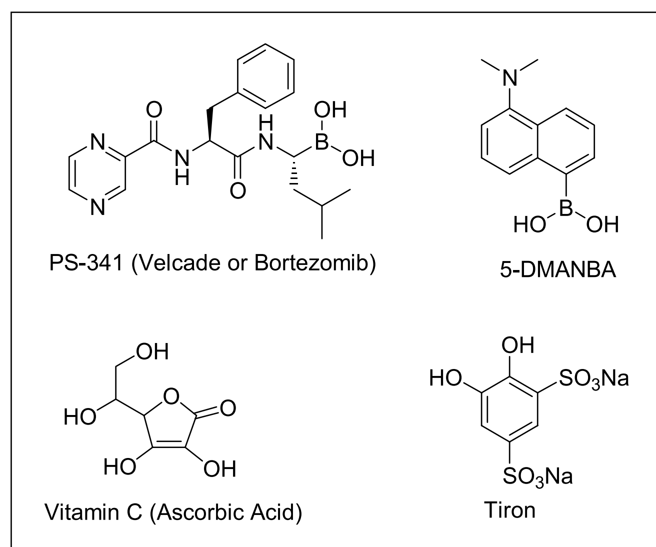

Fig. 1. Chemical structures of PS-341, vitamin C, Tiron, and 5-DMANBA.

antioxidants, suggesting that the inhibition by vitamin C is not related to its effect as an antioxidant. The potential mechanism underlying the interaction between vitamin C and PS-341 was also studied.

Materials and Methods

Reagents. PS-341 was provided by Millennium Pharmaceuticals (Cambridge, MA). It was dissolved in DMSO at a concentration of 10 mmol/L and aliquots were stored at −80°C. Stock solutions were diluted to the desired final concentrations with growth medium just before use. Vitamin C, butylated hydroxyanisole, *N*-acetylcysteine, glutathione, and Tiron were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-(4-Hydroxyphenyl)retinamide was purchased from EMD Biosciences, Inc. (La Jolla, CA). 2′,7′-Dichlorofluorescin diacetate was purchased from Molecular Probes (Eugene, OR). *N*-Succinyl-Leu-Leu-Val-Tyr-amido-4-methyl-coumarin (Ys substrate) was purchased from Biomol (Plymouth Meeting, PA). Human recombinant tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) was purchased from PeproTech, Inc. (Rocky Hill, NJ).

Cell lines and cell culture. SqCC/Yq1 cells were kindly provided by Dr. R. Lotan (M.D. Anderson Cancer Center, Houston, TX). The rest of the cancer cell lines used in the study were purchased from the American Type Culture Collection (Manassas, VA). They were grown in monolayer culture in RPMI 1640 supplemented with 5% fetal bovine serum at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

Cell survival assay. Cells were seeded in 96-well cell culture plates and treated on the 2nd day with the test agents. After a given time, cell number was estimated by the sulforhodamine B assay as previously described (16). Cell survival was calculated by using the following equation: % cell survival = $(A_t / A_c) \times 100$, where A_t and A_c represent the absorbance in treated and control cultures, respectively.

Detection of apoptosis. Apoptotic cells were detected by Annexin V staining using Annexin V-phycoerythrin apoptosis detection kit purchased from BD Bioscience (San Jose, CA) following the instructions of the manufacturer.

Cell cycle analysis and western blot analysis. Flow cytometry was used to analyze cell cycle distribution as previously described (17). The procedures for preparation of whole-cell protein lysates and for the Western blotting were previously described (18). Whole-cell protein lysates (50 μg) were electrophoresed through 10% or 12% denaturing polyacryamide slab gels and transferred to an Immun-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) by electroblotting. The blots were probed or reprobed with the primary antibodies and then antibody binding was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL) according to the protocol of the manufacturer. Rabbit polyclonal antibodies against caspase-8 and poly(ADP-ribose) polymerase were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Mouse monoclonal anti-caspase-3 antibody was purchased from Imgenex (San Diego, CA). Rabbit polyclonal anti-actin antibody was purchased from Sigma Chemical.

Detection of intracellular reactive oxygen species generation. The intracellular reactive oxygen species (ROS) generation was measured using 2′,7′-dichlorofluorescin diacetate assay as previously described (19).

Fluorometric measurement of 20S proteasome activity. Cells at a density of 5,000 per well were seeded in a 96-well plate. On the 2nd day, cells were treated with the test agents. After incubation for 12 hours, the cells were subjected to 20S proteasome activity assay as previously described (20) with slight modifications. In brief, the medium was carefully removed and the cells were washed with PBS. Cells were then lysed by adding 25 μL of 5 mmol/L EDTA to each well and frozen at −80°C overnight. On the 2nd day, 100 μL of substrate buffer (20 mmol/L HEPES, 0.5 mmol/L EDTA, 0.035% SDS, pH 8.0) containing 50 μmol/L Ys substrate were added to each well. After incubation at 37°C for 30 minutes, plate was read at the excitation wavelength 380 nm and emission wavelength 460 nm.

Determination of chemical interaction between a model boronic acid and vitamin C or Tiron. The fluorescent model boronic acid 5-dimethylaminonaphthaleneboronic acid (5-DMANBA; Fig. 1) was used to test the binding between boronic acid and vitamin C or Tiron. 5-DMANBA at 1.12×10^{-5} mol/L in phosphate buffer solution (pH 7.4) was mixed with aqueous vitamin C or Tiron phosphate buffer solution (pH 7.4, 0.1 mol/L) at various concentrations ranging from 0.1 to 200 mmol/L. The fluorescence intensity was then recorded. The binding constant or K_d was calculated using well-established method as previously described (21).

Determination of chemical binding between vitamin C and PS-341. Alizarin red S was used as a reporter compound to measure binding constant between vitamin C and PS-341 as previously described (22, 23). The binding constant was assayed in serum-free RPMI 1640. In principle, the binding of a boronic acid (e.g., PS-341) to alizarin red S induces a large fluorescent intensity increase, which is used to determine the binding constant between PS-341 and alizarin red S. Addition of vitamin C then shifts the equilibrium through the formation of PS-341-vitamin C complex and release of alizarin red S, which reverts alizarin red S to its original nonfluorescent state. Such a shift will allow us easily and accurately determine the binding constant between vitamin C and PS-341. The detailed chemical parts of the assays will be published elsewhere.[3]

Statistical analysis. Cell survival, proteasome activity, and ROS generation between two groups were analyzed with two-sided unpaired Student's *t* tests when the variances were equal or with Welch's corrected *t* test when the variances were not equal by use of GraphPad InStat 3 software (GraphPad Software, Inc., San Diego, CA). The assumption for use of the *t* tests was calculated and suggested by the same software. In all statistical analyses, results were considered to be statistically significant at $P < 0.05$.

Results

Effects of vitamin C and other antioxidants on PS-341-induced apoptotic death in human cancer cells. It was reported that PS-341 promoted ROS generation, which contributed to PS-341-induced apoptosis in some human cancer cell lines

[3] N. Lin and B. Wang, manuscript in preparation.

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(24–26). If this is the case, we speculated that many antioxidants would protect cells from PS-341-induced apoptotic cell death. Therefore, we first examined the effects of several antioxidants with different properties, including vitamin C, on PS-341-induced apoptotic death in two human lung cancer cell lines. Among the tested antioxidants, *N*-acetylcysteine, butylated hydroxyanisole, and glutathione failed to protect cells from PS-341-induced cell death in both cell lines. However, vitamin C inhibited PS-341-induced death (Fig. 2A). Furthermore, we found that PS-341 alone induced cleavage of caspase-8, caspase-3, and poly(ADP-ribose) polymerase (Fig. 2B). In the presence of vitamin C, PS-341 lost its ability to cleave these proteins. However, both *N*-acetylcysteine and butylated hydroxyanisole did not exert any protective effects on PS-341-induced cleavage of caspases (Fig. 2B). Thus, it seems that vitamin C is the only antioxidant of these tested agents that protect cells from PS-341-induced apoptotic death. By Annexin V staining, we also showed that vitamin C abrogated PS-341-induced increase in both apoptotic and necrotic cell populations (Fig. 2C), further confirming the protective effect of vitamin C on PS-341-induced apoptosis. These findings thus showed that vitamin C protects cells from PS-341-induced apoptosis, likely independent of its antioxidative activity.

Furthermore, we examined the effects of vitamin C at concentrations ranging from 62.5 to 500 μmol/L on PS-341-induced cell death in other types of human cancer cell lines. As shown in Fig. 3A, vitamin C at 62.5 μmol/L was able to significantly inhibit the effects of PS-341 on decreasing cell survival in all of the tested cell lines. When its concentration was increased to 125 μmol/L (e.g., PC-3) or 250 μmol/L (e.g., DU145, SqCC/Y1, and HeLa), PS-341 almost completely lost its activity to decrease cell survival. Therefore, it seems that suppression of PS-341 activity by vitamin C is not a cell line–specific event.

The above experiments were all done under the conditions that vitamin C and PS-341 were concurrently added to the cell culture. To have more clues about the mechanism by which vitamin C protects cells from PS-341-induced apoptotic death, we further examined the protective effects of vitamin C on PS-341-induced cell death when it was given either before or after PS-341 treatment. When we first treated cells with PS-341 for a given time, washed away the PS-341, and then fed cell with fresh medium containing vitamin C, we found that the number of survival cells decreased as the exposure time to PS-341 was increased from 2 to 10 hours. However, vitamin C, when added after cells were exposed to PS-341 for a given time, failed to rescue PS-341-initiated cell death (Fig. 3B). If cells were pretreated with vitamin C (2-10 hours), had vitamin C washed away, and then exposed to PS-341, we found that vitamin C did not protect cells from PS-341-induced cell death either (Fig. 3C). These results indicate that vitamin C exerts its protective effect on PS-341-induced apoptosis only when it is added to cells concurrently with PS-341.

Effect of vitamin C on PS-341-mediated augmentation of TRAIL-induced Apoptosis. It has been shown that PS-341 augments TRAIL-induced apoptosis in some cancer cell lines (27–30). In our study, PS-341 alone and TRAIL alone at the tested concentrations decreased cell survival by <20% in both A549 and H1792 cells. However, the combination of PS-341 with either 20 or 10 ng/mL of TRAIL resulted in a decrease of cell survival by >70%, which was more than the sum of cell survival decreases caused by either single agent (Fig. 4A). The decrease of cell survival was due to apoptotic cell death evidenced by activation of caspases and increase in DNA fragmentation (data not shown). Therefore, it seems that PS-341 synergizes with TRAIL to induce apoptotic cell death. However, the addition of vitamin C completely prevented cells from death caused by the combination of PS-341 and TRAIL (Fig. 4A and B). Thus, we

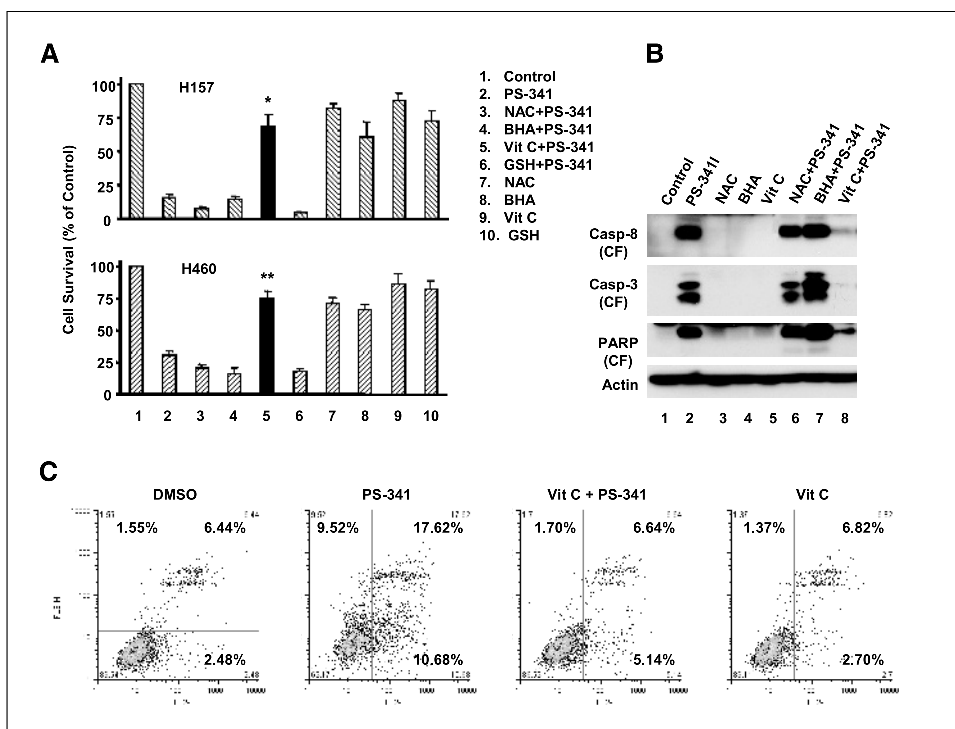

Fig. 2. Effects of vitamin C and other antioxidants on PS-341-induced decrease of cell survival (*A*), caspase activation (*B*), and apoptotic death (*C*) in human lung cancer cells. *A,* the indicated cell lines in 96-well plates were treated with the indicated agents either alone or in combination. After 48 hours, cells were subjected to estimation of cell numbers using the sulforhodamine B assay. Columns, mean of four replicate determinations; bars, SD. *, $P = 0.0014$; **, $P < 0.0001$, compared with PS-341 alone treatment (*lane 2*). *B,* H157 cells were treated with the indicated agents either alone or in combination. After 24 hours, cells were subjected to preparation of whole-cell protein lysates and detection of caspase activation using Western blot analysis. CF, cleaved form. In all of the above studies, the concentrations of PS-341, vitamin C, *N*-acetylcysteine (*NAC*), butylated hydroxyanisole (*BHA*), and glutathione were 50 nmol/L, 500 μmol/L, 5 mmol/L, 500 μmol/L, and 5 mmol/L, respectively. *C,* H157 cells were treated with DMSO control, 50 nmol/L PS-341, 250 μmol/L vitamin C, and vitamin C plus PS-341 for 24 hours. The cells were then subjected to Annexin V staining and flow cytometric analysis. Bottom and top right windows, early and late apoptotic cells, respectively; top left window, necrotic cells.

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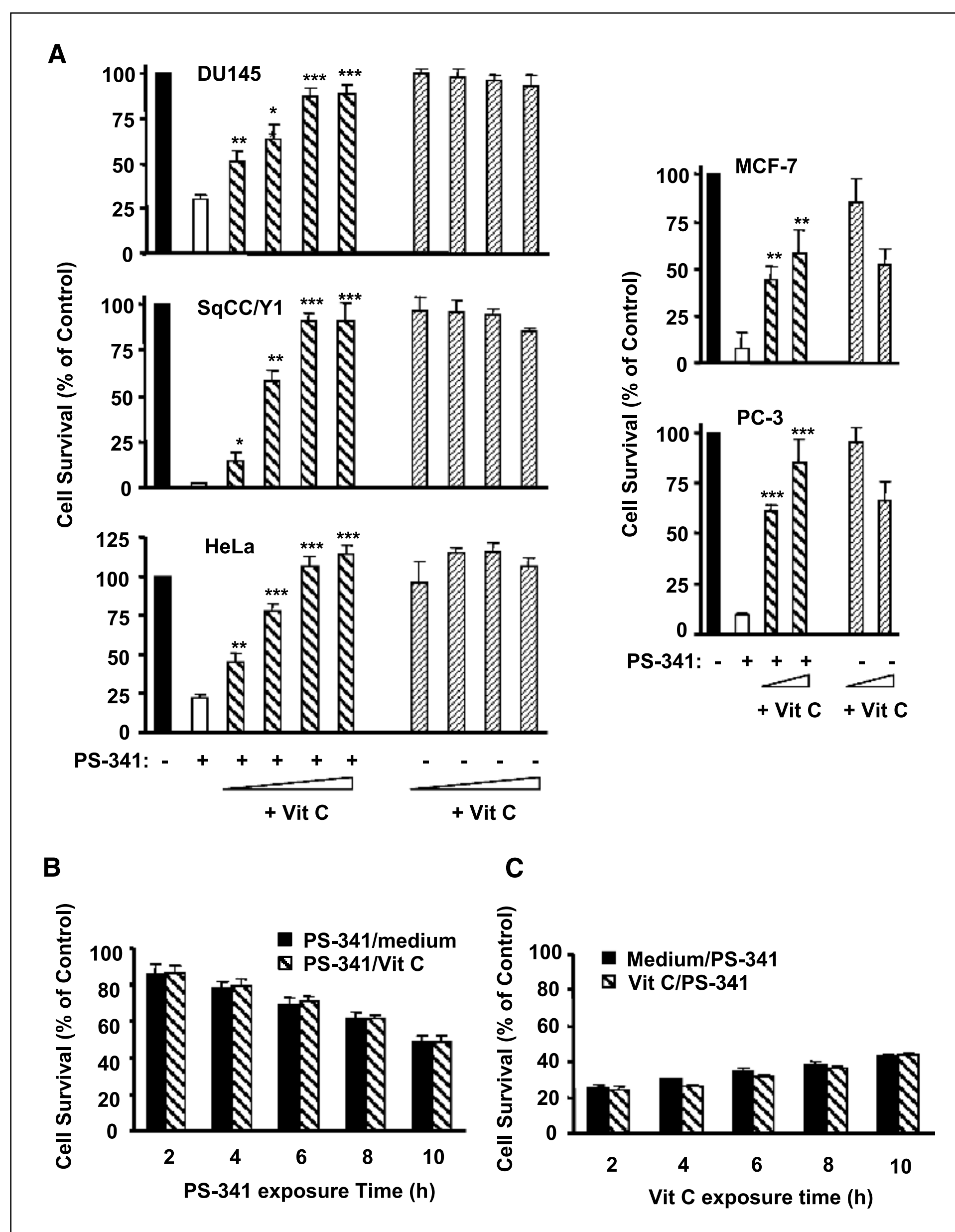

Fig. 3. Protective effects of vitamin C on PS-341-induced cell death in various human cancer cell lines. *A,* the indicated cell lines seeded in 96-well plates were treated with 50 nmol/L PS-341 alone, different concentrations of vitamin C alone, and vitamin C plus PS-341. After 48 hours, cells were subjected to estimation of cell numbers using the sulforhodamine B assay. The concentrations of vitamin C in (*A*) were 62.5, 125, 250, and 500 μmol/L, respectively, and in (*B*) were 62.5 and 125 μmol/L, respectively. *B,* H157 cells in a 96-well plate were treated with 50 nmol/L PS-341 for the given times as indicated, washed once with medium, and then fed with fresh medium containing 250 μmol/L vitamin C. Forty-eight hours later after the initial PS-341 treatment, the cells were subjected to the sulforhodamine B assay. *C,* H157 cells in a 96-well plate were treated with 250 μmol/L vitamin C for the given times as indicated, washed once with medium, and then fed with fresh medium containing 50 nmol/L PS-341. Forty-eight hours later after the initial vitamin C treatment, the cells were subjected to the sulforhodamine B assay. Columns, mean of four replicate determinations; bars, SD. *, $P = 0.01$; **, $P < 0.001$; ***, $P < 0.0001$, compared with PS-341 alone treatment.

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conclude that vitamin C also abrogates the ability of PS-341 to augment TRAIL-induced apoptotic cell death.

Effect of vitamin C on PS-34-induced alteration in cell cycle. It has been shown that PS-341 generally causes G_2-M cell cycle arrest (20, 30). In our study, we obtained a similar result that PS-341 induced G_2-M growth arrest in H157 cells (from 21.8% in DMSO-treated cells to 27.8% in PS-341-treated cells; Fig. 5). In the presence of vitamin C, PS-341 failed to arrest cells at G_2-M phase (25.8% G_2-M cells; Fig. 5). This result indicates that vitamin C abrogates PS-341 effects on altering cell cycle distribution as well.

Effects of PS-341 on intracellular ROS generation. Because several antioxidants failed to protect cells from PS-341-induced apoptosis, we questioned whether PS-341 promoted generation of intracellular ROS. Therefore, we next examined intracellular ROS generation in cells treated with PS-341. *N*-(4-Hydroxyphenyl)retinamide, as a positive control that is known to induce intracellular ROS generation (19, 31), increased ROS generation. PS-341 at 50 nmol/L, which is the effective concentration in inducing apoptotic death, did not significantly increase ROS generation (Fig. 6A). This result, combined with the data that several antioxidants failed to protect cells from PS-341-induced apoptosis as presented above, indicates that ROS generation is unlikely to be the mechanism by which PS-341 induces apoptosis, at least in the cell lines tested.

Effect of vitamin C on PS-341-mediated inhibition of proteasome activity. The antitumor efficacy of PS-341 is tightly associated with its proteasome inhibitory activity (1). Therefore, we examined whether vitamin C interfered with the proteasome inhibitory activity of PS-341. As shown in Fig. 6B, PS-341 indeed inhibited the activity of 20S proteasome as it is supposed to do. However, in the presence of vitamin C, the effect of PS-341 on proteasome inhibition was significantly

inhibited ($P < 0.0001$). Butylated hydroxyanisole and *N*-acetylcysteine, which do not protect cells from PS-341-induced apoptotic death, did not affect PS-341 proteasome inhibitory activity at all. Thus, it seems that vitamin C interferes with the ability of PS-341 to inhibit proteasome activity by a mechanism not related to ROS.

Direct interaction between vitamin C and PS-341. PS-341 is a dipeptidyl boronic acid and boronic acids are known to bind to compounds with the diol functional group through the formation of a tight but reversible complex (32, 33). Vitamin C in its cyclic form has a vicinal diol group on the same plane (attached to a double bond; Fig. 1). Because the dihedral angle of a diol is known to be correlated with its affinity to the boronic acid moiety, we expected that vitamin C would have a high affinity for boronic acid compounds because of its small dihedral angle. Therefore, we speculated that vitamin C might inactivate PS-341 via formation of an inactive complex between vitamin C and PS-341 (Fig. 7A). To test this hypothesis, we determine the binding constant between vitamin C and a model boronic acid called 5-DMANBA (Fig. 1). This model compound was chosen because it changes fluorescent intensity on diol binding (34), which gives a very easy way to determine the binding constant. As expected, vitamin C could bind to 5-DMANBA with an association constant of 10,280 $(\text{mol/L})^{-1}$ (K_d = 97 μmol/L). This means that at about 100 μmol/L of vitamin C, 50% of the boronic acid would be in the binding complex. Following this experiment, we determined whether PS-341 itself indeed chemically bound to vitamin C. Because PS-341 itself does not exhibit fluorescent change, we used alizarin red S as a probe, which exhibits fluorescent change on binding to boronic acids, to determine the binding constant between vitamin C and PS-341 in a competition manner. We detected that vitamin C indeed bound to PS-341 and the binding constant was 1,551 $(\text{mol/L})^{-1}$ (K_d = 645 μmol/L). Collectively, these results show a direct chemical interaction between vitamin C and PS-341.

Effect of Tiron on PS-341-induced cell death and its interaction with PS-341. In the literature, Tiron (Fig. 1) was reported to protect cells from PS-341-induced apoptosis (24, 26). In our study, we also found that Tiron at ≥500 μmol/L concentrations protected cells from PS-341-induced cell death (Fig. 7B), thus confirming the findings by other groups (24, 26). It should be noted that Tiron also has a pair of vicinal diol group with a very small dihedral angle (Fig. 1). Thus, we speculated that Tiron might also abrogate PS-341 activity through a mechanism similar to that of vitamin C by forming an inactive complex with PS-341 (Fig. 7A). Therefore, we examined the binding of Tiron with 5-DMANBA and found that the binding constant between Tiron and 5-DMANBA was 13,352 $(\text{mol/L})^{-1}$ with a K_d of 75 μmol/L. Such results suggest that the protective effect of Tiron on PS-341-mediated apoptosis may also be related to its binding to the boronic acid moiety of PS-341.

Discussion

Human plasma levels of vitamin C are typically 50 to 70 μmol/L whereas tissue concentrations range from around 1 mmol/L for skeletal and smooth muscle to >10 mmol/L in leukocytes, brain, adrenal glands, and lung (35). In the clinical practice for cancer treatment, vitamin C at a dose of 1.25 g, administered orally and i.v., produced mean peak plasma concentrations of ~135 and 900 μmol/L, respectively (36). In this study, our data clearly indicate that vitamin C at the tested concentrations ranging from 62.5 to 500 μmol/L suppresses or abrogates the ability of PS-341 to induce apoptosis and growth arrest and to augment TRAIL-induced apoptosis in human cancer cells. PS-341 is Food and Drug Administration approved for the treatment of patients with

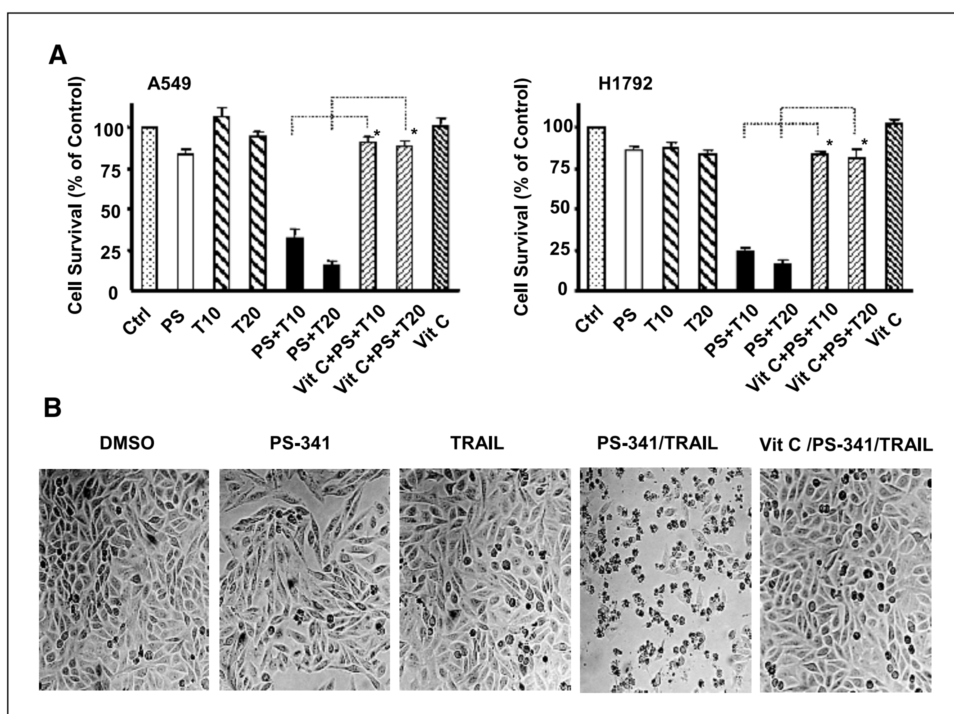

Fig. 4. Effects of vitamin C on PS-341-mediated augmentation of TRAIL-induced apoptosis. *A,* the indicated cell lines seeded in 96-well plates were treated with 50 nmol/L PS-341 alone, 10 or 20 ng/mL TRAIL alone, 200 μmol/L vitamin C alone, PS-341 combined with TRAIL, and vitamin C combined with PS-341 plus TRAIL. After 19 hours, cells were subjected to estimation of cell numbers using the sulforhodamine B assay. Ctrl, solvent control; PS, PS-341; T10, 10 ng/mL TRAIL; T20, 20 ng/mL TRAIL; Columns, mean of four replicate determinations; bars, SD. *, $P < 0.0001$. *B,* A549 cells were treated with 50 nmol/L PS-341 alone, 20 ng/mL TRAIL alone, 200 μmol/L vitamin C alone, PS-341 combined with TRAIL, and vitamin C combined PS-341 plus TRAIL. After 19 hours, photos were taken under an inverted microscope.

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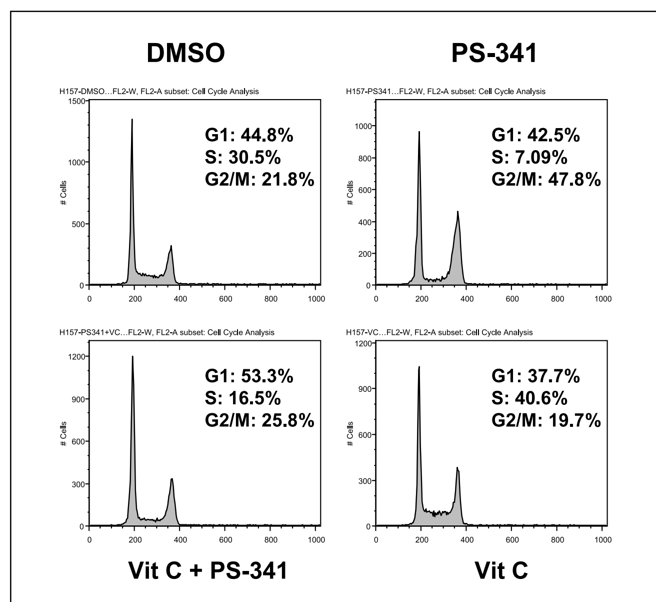

Fig. 5. Effect of vitamin C on PS-341-induced G_2-M cell cycle arrest. H157 cells were treated with DMSO control, 50 nmol/L PS-341, 250 μmol/L vitamin C, and vitamin C plus PS-341 for 24 hours. The cells were then subjected to cell cycle analysis by a flow cytometry.

relapsed multiple myeloma and has shown promising activity in tumors, such as lymphoma, either alone or in combination with other agents. Currently, there are multiple clinical trials that test PS-341 efficacies in treatment of various types of cancer either as a single agent and in combination with other agents. Additionally, many patients are routinely taking vitamin supplements or extra vitamin C for health-related reasons. Our findings have clearly shown a direct chemical interaction between vitamin C and PS-341. Thus, precautions should be taken when using PS-341 in the clinic to avoid a potential adverse drug interaction for cancer treatment. Our study also indicates that vitamin C inactivates PS-341 only when given concurrently. Thus, one way to prevent such an interaction is to avoid taking PS-341 and vitamin C concurrently.

It seems that vitamin C has high concentrations of distributions in normal tissues or organs such as skeletal and smooth muscle, leukocytes, brain, adrenal glands, and lung (35). Our findings, on the other hand, may have good implications for clinical application of PS-341. High concentrations of vitamin C in some normal organs or tissues may prevent them from potential harm caused by PS-341-mediated toxicities or side effects. PS-341 is well tolerated in cancer patients whereas it is assumed to be toxic because it may interfere with and alter functions of multiple cellular proteins due to its wholesale inhibition of proteasome activity. Due to oxidative stress, tumor tissues may have relatively low levels of vitamin C whereas many normal organs or tissues have very high levels of vitamin C. Thus, it is possible that high levels of vitamin C in normal organs or tissues minimize or neutralize PS-341 potential toxicities while maintaining its anticancer activity.

It has been claimed that PS-341 induces apoptosis through promotion of ROS generation (24–26). In our study, we failed to detect any increase in ROS generation in cells exposed to PS-341. Although vitamin C is an antioxidant (15) and inhibits ROS generation (20), the other antioxidants, butylated hydroxyanisole, *N*-acetylcysteine, and glutathione, did not protect cells from PS-341-induced apoptosis at all. Mannitol is another antioxidant and it has been used to formulate PS-341 in the clinic. Mannitol at up to 10 mmol/L did not exert a

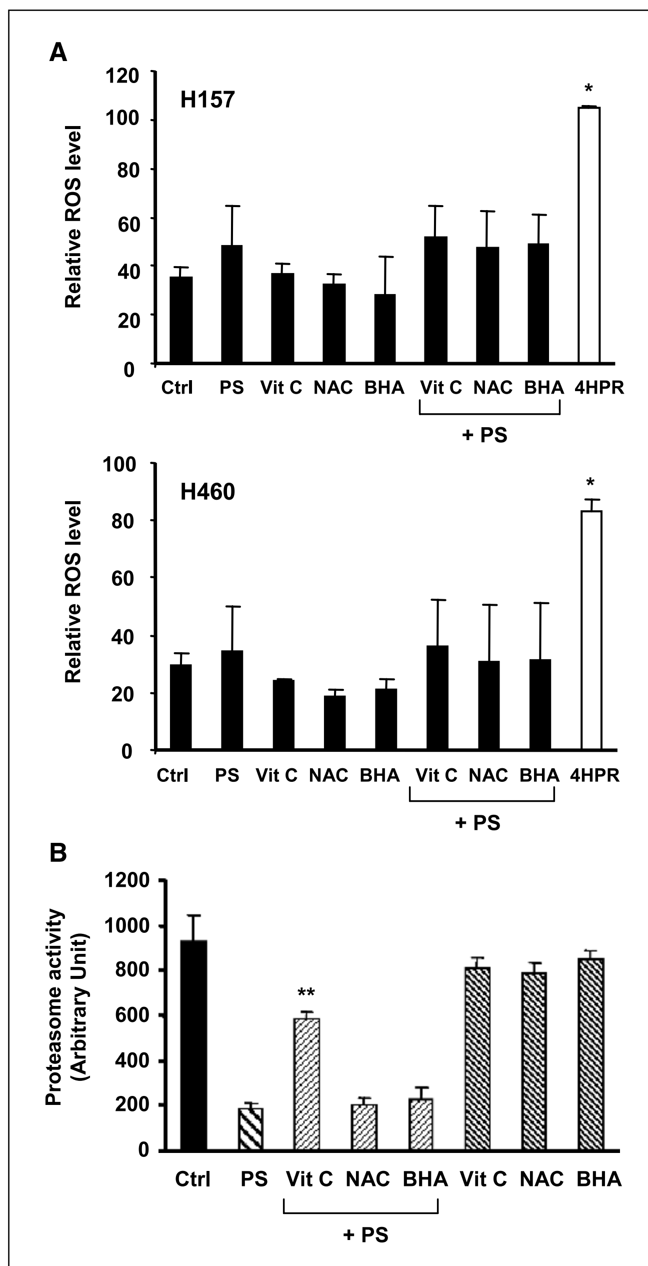

Fig. 6. Effects of vitamin C on ROS generation (*A*) and PS-341-mediated proteasome inhibition (*B*). *A,* the indicated human lung cancer cell lines were loaded with 2′,7′-dichlorofluorescin diacetate and then exposed to the indicated treatments for 3 hours. ROS generation was then determined using the 2′,7′-dichlorofluorescin diacetate assay. The concentrations for PS-341, vitamin C, *N*-acetylcysteine, butylated hydroxyanisole, and *N*-(4-hydroxyphenyl) retinamide (*4HPR*) were 50 nmol/L, 500 μmol/L, 5 mmol/L, 500 μmol/L, and 10 μmol/L, respectively. *B,* H157 human lung cancer cells were exposed for the indicated treatments for 8 hours. The proteasome activity was then determined as described in Materials and Methods. The concentrations for PS-341, vitamin C, *N*-acetylcysteine, and butylated hydroxyanisole were 50 nmol/L, 500 μmol/L, 5 mmol/L, and 500 μmol/L, respectively. Columns, mean of four replicate determinations; bars, SD. *, $P < 0.01$; **, $P < 0.0001$, compared with PS-341 alone treatment (*lane 2*).

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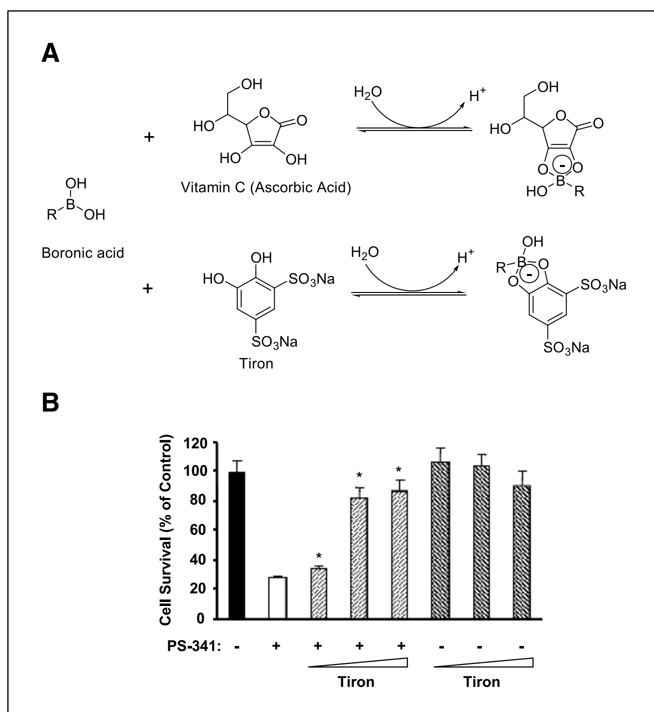

Fig. 7. Potential binding between boronic acids and vitamin C or Tiron (*A*) and the effect of Tiron on PS-341-induced cell death (*B*). *A,* potential interaction between boronic acids and vitamin C or Tiron. R can be other structures linked to boronic acid such as PS-341. *B,* H157 cells seeded in a 96-well plate were treated with 50 nmol/L PS-341 alone, Tiron alone (100, 500, and 1,000 μmol/L), and Tiron combined with PS-341. After 48 hours, cells were subjected to estimation of cell numbers using the sulforhodamine B assay. Columns, mean of four replicate determinations; bars, SD. *, $P < 0.001$, compared with PS-341 alone treatment.

protective effect on PS-341-induced cell death.[4] Collectively, we suggest that PS-341 is unlikely to induce apoptosis via promotion of ROS generation, at least in our systems. Correspondingly, we suggest that vitamin C protects cells from PS-341-induced apoptosis independent of its antioxidant activity.

In our study, we found that vitamin C, when given concurrently with PS-341, inhibited or abrogated all the tested biological activities caused by PS-341 including induction of apoptosis and G_2-M arrest, enhancement of TRAIL-induced apoptosis, and inhibition of proteasome activity. Moreover, vitamin C failed to exert protective effects on PS-341-induced apoptosis when it was given to cells either before or after PS-341 treatment. Therefore, we were wondering whether vitamin C directly interacted with PS-341 leading to inactivation of PS-341. It is known that boronic acids bind to compounds with the diol functional group through the formation of a tight but reversible complex (33, 34). Such interactions have been used for the synthesis of sensors for sugars (37, 38) as well as for other biological applications (39–42). PS-341 is a dipeptidyl boronic acid whereas vitamin C has adjacent hydroxyl groups. Therefore, we speculated that vitamin C could conceivably form a complex with PS-341, leading to inactivation of PS-341. Using a model boronic acid, we confirmed the binding between vitamin C and boronic acid by measuring the binding constant. Moreover, we indeed detected a direct chemical binding between vitamin C and PS-341. Collectively, these results indicate that vitamin C can chemically form a complex with PS-341. The K_d for vitamin C to bind to PS-341 in the tested buffer system is 645 μmol/L, which is close to the concentration (i.e., 500 μmol/L) of vitamin C that substantially inactivates PS-341 in cell cultures. Therefore, we conclude that vitamin C interferes with the biological activity of PS-341 by forming a biologically inactive complex with PS-341. This complex is not only inactive but also expected to have very poor membrane permeability that would prevent the drug from entering into the cell. In this way, we may explain why vitamin C abolished all of the tested biological activities mediated by PS-341 in human cancer cells.

In the studies that claimed the role of ROS generation in mediating PS-341-induced apoptosis, Tiron was the only antioxidant used to probe the involvement of ROS (24, 26). In agreement with their findings, we also found that Tiron protected cells from PS-341-induced cell death. However, it should be noted that Tiron also has a pair of vicinal diols with a very small dihedral angle, which is an important property for strong binding with a boronic acid moiety (Fig. 1). This suggests that Tiron may also bind to boronic acids too. Indeed, we showed that Tiron binds to the model boronic acid with very high affinity. Therefore, it is plausible to argue that Tiron, instead of working as an antioxidant in this particular case, is likely to abrogate PS-341-induced apoptosis via formation of a biological inactive complex with PS-341 as vitamin C does.

In summary, this study has shown that vitamin C inactivates PS-341, likely by forming a biological inactive complex with PS-341. Therefore, the negative effect of vitamin C on the anticancer activity of PS-341 should be considered when PS-341 is used in the clinic for cancer treatment.

[4] W. Zou and S.Y. Sun, unpublished data.

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