The Proteasome Inhibitor PS-341 (Bortezomib) Up-Regulates DR5 Expression Leading to Induction of Apoptosis and Enhancement of TRAIL-Induced Apoptosis Despite Up-Regulation of c-FLIP and Survivin Expression in Human NSCLC Cells

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Abstract
The proteasome inhibitor PS-341 (bortezomib or Velcade), an approved drug for treatment of patients with multiple myeloma, is currently being tested in clinical trials against various malignancies, including lung cancer. Preclinical studies have shown that PS-341 induces apoptosis and enhances tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–induced apoptosis in human cancer cells with undefined mechanisms. In the present study, we show that PS-341 up-regulated caspase-8–dependent apoptosis, cooperated with TRAIL to induce apoptosis, and up-regulated death receptor 5 (DR5) expression in human non–small cell lung cancer (NSCLC) cells. DR5 induction correlated with the ability of PS-341 to induce apoptosis. Blockage of PS-341–induced DR5 up-regulation using DR5 small interfering RNA (siRNA) rendered cells less sensitive to apoptosis induced by either PS-341 or its combination with TRAIL, indicating that DR5 up-regulation mediates PS-341–induced apoptosis and enhancement of TRAIL-induced apoptosis in human NSCLC cells. We exclude the involvement of c-FLIP and survivin in mediating these events because c-FLIP (i.e., FLIPr) and survivin protein levels were actually elevated on exposure to PS-341. Reduction of c-FLIP with c-FLIP siRNA sensitized cells to PS-341–induced apoptosis, suggesting that c-FLIP elevation protects cells from PS-341–induced apoptosis. Thus, the present study highlights the important role of DR5 up-regulation in PS-341–induced apoptosis and enhancement of TRAIL-induced apoptosis in human NSCLC cells.

Introduction
The tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) receptor death receptor 4 (DR4; also called TRAIL-R1) and death receptor 5 (DR5; also named Apo2, TRAIL-R2, TRICK2, or Killer/DR5) belong to the TNF receptor gene superfamily, all of which share a similar, cysteine-rich extracellular domain and additional cytoplasmic death domain (1). Both DR4 and DR5, located at the cell surface, become activated or trimerized on binding to their ligand TRAIL or overexpression and then signal apoptosis through caspase-8–mediated activation of caspase cascades (1). Recently, these death receptors have attracted much more attention because their ligand TRAIL preferentially induces apoptosis in transformed or malignant cells, showing potential as a tumor-selective apoptosis-inducing cytokine for cancer treatment. The expression of DR4 and DR5 is inducible by certain stimuli, including some cancer therapeutic agents (2, 3). It has been documented that induction of DR4 and/or DR5 accounts for induction of apoptosis and/or enhancement of TRAIL-induced apoptosis by certain cancer therapeutic agents (4–9).

Caspase-8 activation is a critical step in initiating death receptor–induced apoptosis (1). c-FLIP is the major protein that prevents caspase-8 from activation by death receptors. Although more than 10 isoforms of c-FLIP mRNA have been described, only 2 of them, FLIPr and FLIPl, have been significantly studied at the protein level (10). Both proteins can be recruited to the death-inducing signaling complex (DISC) to inhibit caspase-8 activation (10, 11). There are an increasing number of studies showing that modulation of c-FLIP levels affects cell sensitivity to death receptor–mediated apoptosis (10, 11).

It is well known that the extrinsic death receptor–mediated pathway can activate the intrinsic mitochondria-mediated pathway, through caspase-8–mediated cleavage or truncation of Bid protein, leading to induction of apoptosis (12). Survivin, a family member of the inhibitor of apoptosis proteins, acts downstream of mitochondria to prevent processing of initiator caspase-9 from the apoptosisome, leading to inhibition of the activity of the effector caspases. Thus, survivin modulates both the extrinsic and the intrinsic apoptotic pathways (13). Many studies have shown that induction of survivin expression causes cellular resistance to drug-induced apoptosis, whereas down-regulation of survivin using various means, such as small interfering RNA (siRNA), either induces apoptosis or sensitizes cells to undergo drug- or death ligand/receptor–induced apoptosis (13).

PS-341 (also called bortezomib or Velcade) is an approved drug for treatment of patients with relapsed multiple myeloma. Currently, there are many ongoing clinical trials that test the anticaner efficacy of PS-341 or its combinations with other agents in different types of cancers, including lung cancer (14, 15). Many preclinical studies documented that PS-341 alone or in combination with other cancer therapeutic agents, including TRAIL, induces apoptosis in a variety of human cancer cells in vitro, including both hematologic and solid tumor malignancies, and inhibits the growth of tumor xenografts in vivo (16, 17). However, the molecular mechanisms underlying PS-341–induced apoptosis and enhancement of apoptosis when combined with other agents, including TRAIL, particularly in human lung cancer...
cells, remain largely uncharacterized, although it seems to be associated with nuclear factor-κB inhibition (14, 18, 19), c-Jun NH₂-terminal kinase (JNK) activation (19–21), or Bik and Bim accumulation (22, 23) shown in certain types of cancer cells.

PS-341 has been shown to sensitize cells to TRAIL-induced apoptosis in certain types of cancer cells; this effect seems to be associated with Bik accumulation or c-FLIP down-regulation (22–24). Other proteasome inhibitors, such as MG132, increase DR5 expression, which mediates induction of apoptosis and enhances TRAIL-induced apoptosis by these inhibitors (25–27). To show the mechanism by which PS-341 induces apoptosis in human non-small cell lung cancer (NSCLC) cells, we studied the effects of PS-341 on the expression of DR4, DR5, c-FLIP, and survivin and their effect on PS-341–induced apoptosis and enhancement of TRAIL-induced apoptosis. Our results show that DR5 up-regulation plays an important role in PS-341–induced apoptosis and enhancement of TRAIL-induced apoptosis in human NSCLC cells.

Materials and Methods

Reagents. The powder of pure PS-341 was provided by Millennium Pharmaceuticals. It was dissolved in DMSO at a concentration of 1 mmol/L, and aliquots were stored at −80°C. Stock solutions were diluted to the desired final concentrations with growth medium just before use. MG132 and epoxomicin, two additional proteasome inhibitors, were purchased from Sigma Chemical Co. and Calbiochem, respectively. Human recombinant TRAIL was purchased from Biomol or PeproTech, Inc. Mouse monoclonal anti-DR4 antibody was purchased from ProSci, Inc. Mouse monoclonal anti-DR5 antibody was purchased from Biomol or PeproTech, Inc. Rabbit polyclonal anti-DR5 antibody (NF6) was purchased from Alexis Biochemicals. Mouse antibody (B-N28) was purchased from Diaclone. Mouse monoclonal anti-DR5 antibody was purchased from ProSci, Inc. Mouse monoclonal anti-DR4 antibody was purchased from ProSci, Inc. Mouse monoclonal anti-DR5 antibody (NF6) was purchased from Alexis Biochemicals. Mouse monoclonal anti-caspase-3 was purchased from Imgenex. Rabbit anti-caspase-8, anti-caspase-9, anticaspase-6, anti-lamin A/C, and anti–poly(ADP-ribose) polymerase (PARP) antibodies and mouse monoclonal anti-survivin antibody were purchased from Cell Signaling Technology, Inc. Rabbit polyclonal anti-j-actin antibody was purchased from Sigma Chemical.

Cell culture. The human NSCLC cell lines used in this study were purchased from the American Type Culture Collection. They were grown in monolayer culture in RPMI 1640 with glutamine (Sigma Chemical) supplemented with 5% fetal bovine serum at 37°C in a humidified atmosphere consisting of 5% CO₂ and 95% air. The immortalized normal human bronchial epithelial cell lines BEAS-2B (28) and HBE3KT (29) were provided by Dr. R. Lotan (M. D. Anderson Cancer Center, Houston, TX) and J.D. Minna (The University of Texas Southwestern Medical Center, Dallas, TX) and cultured as described previously (28, 29).

Cell survival assay. Cell survival was estimated by sulforhodamine B (SRB) assay as described previously (30).

Western blot analysis. Preparation of whole-cell protein lysates and the procedures for the Western blotting were described previously (4).

Detection of apoptosis. The amounts of cytoplasmic histone-associated DNA fragments (mononucleosome and oligonucleosomes) formed during apoptosis were measured using a Cell Death Detection ELISA™ kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. The sub-G₁ population was analyzed using flow cytometry as described previously (31). In addition, caspase activation and their substrate cleavage were also detected by Western blot analysis as described above as another indicator of apoptosis.

Detection of cell surface death receptors. The procedure for direct antibody staining and subsequent flow cytometric analysis of cell surface protein was described previously (32). The mean fluorescence intensity (MFI) that represents antigenic density on a per cell basis was used to represent DR5 expression level. Phycocerythrin-conjugated mouse anti-human DR5 (DJR2-4) and anti-human DR4 (DJR1) monoclonal antibodies and phycoerythrin mouse IgG1 isotype control (MOPC-21/P3) were purchased from eBioscience.

Gene silencing using siRNA. The siRNA duplexes for control, caspase-8, DR4, and DR5 genes were described previously (4, 5). c-FLIP siRNA duplex

Figure 1. PS-341 induces DNA fragmentation (A), caspase activation (B), and caspase-8–dependent apoptosis (C and D). A, the indicated cell lines were treated with 50 nmol/L PS-341 for 24 h and then subjected to evaluation of DNA fragmentation. B, the indicated cell lines were treated with the given concentrations of PS-341 for 24 h and then subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis. C and D, H157 cells were cultured in a 24-well plate and on the 2nd day transfected twice with control (Ctrl) or caspase-8 (Casp-8) siRNA with a 48-h interval between transfections. Forty hours after the second transfection, cells were treated with 50 nmol/L PS-341. The cells were either harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis after an 8-h treatment (C) or subjected to estimation of DNA fragmentation using the Cell Death Detection ELISA kit after a 24-h treatment (D). Columns, mean of triplicate determinations; bars, SD. CF, cleaved fragment. NSB, nonspecific band.
targeting the sequence 5'-AATTCTCCGAACGTGTCACGT-3' of c-FLIP gene (+514 to +534) was described previously (33). Transfection of these siRNA duplexes was conducted in 24-well or 96-well plates using the HiPerFect transfection reagent (Qiagen) following the manufacturer’s manual. Gene silencing effects and caspase activation were evaluated by Western blot analysis, whereas DNA fragmentation and cell survival were measured by a Cell Death Detection ELISA Plus kit and the SRB assay, respectively, as described above.

Results

**PS-341 induces caspase-8–dependent apoptosis.** To understand the mechanism by which PS-341 induces apoptosis in human NSCLC cells, we first determined the effects of PS-341 on induction of apoptosis by measuring DNA fragmentation in several NSCLC cell lines. As shown in Fig. 1A, PS-341 increased DNA fragmentation in H157, H460, and H1792 cell lines, indicating that these cell lines undergo apoptosis on PS-341 treatment. The sensitivities to undergo apoptosis of these cell lines are H1792 > H460 > H157. We also determined the effects of PS-341 on induction of apoptosis in BEAS-2B and HBEC3KT immortalized normal human bronchial epithelial cells. Compared with NSCLC cell lines, these normal cell lines were much less sensitive to PS-341–induced apoptosis (Fig. 1A).

We next determined the effects of PS-341 on activation of different caspases in the above human NSCLC cell lines. PS-341 at the tested concentration range (25–100 nmol/L) effectively induced cleavage of caspase-8, caspase-9, caspase-3, and PARP as indicated by appearance of the cleaved forms of these proteins in H157, H460, and H1792 cells treated with PS-341, showing that PS-341 activates these caspases (Fig. 1B). Moreover, it seemed that the degree of caspase-8 activation, but not caspase-9 activation, by PS-341 was associated with cell sensitivities to undergo cleavage of caspase-3 and PARP and DNA fragmentation (Fig. 1B), suggesting that caspase-8 activation may be important in PS-341–induced apoptosis. Thus, we further determined whether caspase-8 activation is required for PS-341–induced apoptosis. To this end, we used caspase-8 siRNA to block caspase-8 activation. As presented in Fig. 1C, the levels of uncleaved caspase-8 in caspase-8 siRNA-transfected cells were substantially decreased compared with those in cells transfected with control siRNA. Accordingly, we detected cleaved forms of caspase-8 in control siRNA-transfected cells, but not in cells transfected with caspase-8 siRNA, on PS-341 treatment, indicating a successful inhibition of caspase-8 activation induced by PS-341. Under these conditions, PS-341 efficiently increased levels of DNA fragments in control siRNA-transfected cells but only weakly in caspase-8 siRNA-transfected cells (Fig. 1D). These results indicate that caspase-8 activation is required for PS-341–induced apoptosis in human NSCLC cells.

**PS-341 cooperates with TRAIL to enhance apoptosis.** Sensitization of TRAIL-induced apoptosis by PS-341 has been
documented in other types of cancer cells but not in lung cancer cells. Thus, we examined the effects of PS-341 in combination with TRAIL on cell survival and apoptosis in several human NSCLC cell lines, including A549, which is relatively resistant to TRAIL-induced apoptosis (34). TRAIL at concentrations ranging from 25 to 100 ng/mL decreased cell survival by \( \leq 25\% \) in A549, H1792, and H157 cells, whereas PS-341 at 50 nmol/L alone decreased cell survival by \( < 30\% \) in A549 and H1792 cells and by \( < 50\% \) in H157 cells. However, the combination of PS-341 and TRAIL achieved 50\% to 80\% decreases in cell survival in A549 and H1792 cells and \( > 90\% \) in H157 cells (Fig. 2A). Similar result was also observed in TRAIL-sensitive H460 cells (Fig. 2A).

By specifically measuring DNA fragmentation, a hallmark of apoptosis, we detected enhanced DNA fragmentation in both H1792 and A549 cell lines treated with the combination of PS-341 and TRAIL (Fig. 2B). For example, TRAIL at 50 ng/mL alone and PS-341 at 50 nmol/L alone exerted limited effects on increasing DNA fragmentation (0.280 and 0.133 arbitrary units, respectively) in A549 cells. However, their combination increased DNA fragmentation up to 1.67 arbitrary unit (Fig. 2B). Collectively, these results clearly indicate that the combination of PS-341 and TRAIL exhibits a more than additive (synergistic) effect on induction of apoptosis in human NSCLC cells. Moreover, we also examined the effects of the combination on caspase activation in these cell lines. As presented in Fig. 2C, the combination of PS-341 and TRAIL was obviously more potent than each single agent in inducing cleavage of caspase-8, caspase-9, caspase-6, and caspase-3 and their substrates PARP and lamin A/C, evidenced by increased amounts of cleaved bands in cells treated with the combination in comparison with those in cells treated with either PS-341 or TRAIL alone. These data further support that the combination of PS-341 and TRAIL enhances apoptosis in human NSCLC cells.

**PS-341 up-regulates the expression of DR5, FLIP\(_S\), and survivin.** Because both caspase-8 activation and enhancement of TRAIL-induced apoptosis involve TRAIL death receptors and c-FLIP, we next examined the effects of PS-341 on the expression of DR4, DR5, and c-FLIP in these NSCLC cell lines. By Western blot analysis, we detected increased and dose-dependent DR5 expression in the three NSCLC cell lines after exposure to PS-341. The degrees of DR5 induction in these cell lines were H1792 > H460 > H157 cells (Fig. 3), which correlate with cell sensitivity to undergo apoptosis and caspase-8 activation as described above (Fig. 1A and B). PS-341 increased DR4 levels in H1792 cells but not in H157 and H460 cells (Fig. 3). In agreement, cell surface DR5 levels were increased in the three cell lines treated with PS-341, whereas cell surface DR4 levels were substantially increased only in H1792 cells (Fig. 3). PS-341 did not apparently alter FLIP\(_S\) levels; instead, it increased FLIP\(_L\) levels in the three cell lines, particularly in H157 and H460 cells (Fig. 3), which were less sensitive to PS-341–induced apoptosis compared with H1792 cells (Fig. 1A). We noted that the basal levels of c-FLIP in H157 and H460 cells were much higher than in H1792 cells (Fig. 3). These results suggest that c-FLIP up-regulation may protect cells from PS-341–induced apoptosis. Together, these results suggest that DR5 up-regulation, rather than c-FLIP modulation, plays an important role in PS-341–mediated apoptosis and enhancement of TRAIL-induced apoptosis in human NSCLC cells.

It is well known that survivin, Bcl-2, and Bcl-X\(_L\) are other important proteins involved in regulating apoptosis or enhancing TRAIL-induced apoptosis (13, 35, 36). Therefore, we also measured the levels of these proteins in cells exposed to PS-341. PS-341 did not alter the expression of either Bcl-2 or Bcl-X\(_L\) in human NSCLC cells even at concentrations up to 1 \( \mu \)mol/L (see Supplementary Fig. S1). In a similar fashion to FLIP\(_L\) modulation, we found that PS-341 also increased the levels of survivin in the tested NSCLC cell lines, particularly in H460 and H1792 cells (Fig. 3). We also examined the effects of two other proteasome inhibitors, MG132 and epoxomicin, on the expression levels of DR5, c-FLIP, and

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Effects of PS-341 as well as other proteasome inhibitors on the expression of DR5, DR4, c-FLIP, and survivin (A and C) and cell surface distributions of death receptors (B) in human NSCLC cell lines. A, the indicated cell lines were treated with the given concentrations of PS-341 for 8 h and then subjected to preparation of whole-cell protein lysates. The given proteins were detected using Western blot analysis. B, the indicated cell lines were treated with 50 nmol/L PS-341 for 12 h and then harvested for analysis of cell surface DR5 and DR4 by immunofluorescent staining and subsequent flow cytometry. Filled gray peaks, cells stained with a matched control phycoerythrin-conjugated IgG isotype antibody; open peaks, cells stained with phycoerythrin-conjugated anti-DR5 or DR4 antibody. The number in each parenthesis is the MFI. C, H460 cells were treated with the indicated concentrations of MG132 or epoxomicin (Epo) for 8 h and then subjected to preparation of whole-cell protein lysates. The given proteins were detected using Western blot analysis. LE, longer exposure.
suggest that elevation of FLIPS and survivin by PS-341 is likely to epoxomicin induced DR5 expression. Collectively, these results or epoxomicin. In addition, we also found that both MG132 and detected increases in survivin levels in cells exposed to either MG132 in cells treated with either MG132 or epoxomicin. Similarly, we also be a consequence of proteasome inhibition.

**DR5 up-regulation contributes to PS-341–induced apoptosis.**

To determine whether DR5 up-regulation is involved in PS-341–induced apoptosis in human NSCLC cells, we used DR5 siRNA to block PS-341–induced up-regulation and then determined its effect on PS-341–induced apoptosis. As presented in Fig. 4A, transfection of DR5 siRNA into H157 cells effectively decreased the basal levels of DR5 expression and PS-341–induced DR5 up-regulation. In this experiment, we also included DR4 silencing as a control, transfection of which into H157 cells substantially reduced the basal levels of DR4 expression. These results indicate successful knockdown of either DR5 or DR4. Under these conditions, DR5 siRNA protected cells from PS-341–induced decrease in cell survival, whereas DR4 siRNA did not have such an effect (Fig. 4B). By measuring caspase activation using Western blot analysis, we found that PS-341 induced cleavage of caspase-8, caspase-9, caspase-3, and PARP in cells transfected with control or DR4 siRNA but not in DR5 siRNA-transfected cells (Fig. 4A). Accordingly, PS-341 increased DNA fragmentation in cells transfected with control or DR4 siRNA, but this effect was drastically inhibited in DR5 siRNA-transfected cells (Fig. 4C). In agreement, we observed that PS-341 also induced less amounts of cleaved forms of caspase-8, caspase-3, and PARP and DNA fragments in DR5 siRNA-transfected H460 cells than in control siRNA- transfected H460 cells (see Supplementary Fig. S2). Together, these results indicate that DR5 up-regulation plays a critical role in mediating PS-341–induced apoptosis in human NSCLC cells.

**DR5 up-regulation contributes to PS-341–mediated enhancement of TRAIL-induced apoptosis.**

Because PS-341 increases FLIP₅ levels, we also determined whether PS-341 enhances TRAIL-induced apoptosis via up-regulation of DR5. The combination of PS-341 and TRAIL exhibited enhanced effects on cleavage of caspase-8, caspase-3, and PARP as indicated by the levels of the cleaved bands by Western blotting, in control siRNA-transfected cells but not in cells transfected with DR5 siRNA (Fig. 5A). Accordingly, the combination of PS-341 and TRAIL was significantly less active in decreasing cell survival (Fig. 5B) and in increasing DNA fragmentation (Fig. 5C) in DR5 siRNA-transfected cells than in cells transfected with control siRNA. Together, these results indicate that PS-341 up-regulates DR5 expression, leading to enhancement of TRAIL-induced apoptosis.

**PS-341 induces apoptosis independently of TRAIL ligand.**

The preceding data clearly indicate that PS-341 induces apoptosis in human NSCLC cells through a DR5-mediated mechanism, whereas others have shown that PS-341 increased TRAIL expression, which contributes to PS-341–induced apoptosis in primary chronic lymphocytic leukemia cells (37). Thus, we further determined if PS-341–induced DR5-dependent apoptosis involves the TRAIL ligand. By Western blot analysis, we observed that H157 cells expressed very low levels of TRAIL, which were not further increased by PS-341 (see Supplementary Fig. S3A). The presence of soluble recombinant DR5:Fc, which neutralizes TRAIL, abolished TRAIL-induced decrease in cell survival and increase in DNA fragmentation but failed to protect cells from PS-341–induced cell death (see Supplementary Figure A4).
Collectively, we conclude that PS-341 induces apoptosis in human NSCLC cells independently of TRAIL.

**Blockage of FLIP\(_\text{s}\) elevation sensitizes cells to PS-341 treatment.** To determine whether FLIP\(_\text{s}\) elevation by PS-341 is associated with cell resistance to PS-341, we used c-FLIP siRNA to block PS-341–induced FLIP\(_\text{s}\) elevation and then examined cell sensitivity to PS-341. As presented in Fig. 6B, transfection of c-FLIP siRNA not only decreased basal levels of c-FLIP (both FLIP\(_\text{a}\) and FLIP\(_\text{s}\)) but also more importantly abrogated PS-341–induced FLIP\(_\text{s}\) elevation. Subsequently, the cell sensitivity to PS-341 treatment in c-FLIP siRNA-transfected cells was greatly increased in comparison with cells transfected with control siRNA (Fig. 6B). In both H157 and H460 cells, PS-341 induced more apoptosis in c-FLIP siRNA-transfected cells than in control siRNA-transfected cells (Fig. 6C). For example, PS-341 induced ~15% apoptosis in control siRNA-transfected cells but 30% apoptosis in c-FLIP siRNA-transfected cells, whereas transfection of c-FLIP siRNA alone caused only ~10% apoptosis (Fig. 6C). Thus, these results have proved our speculation that FLIP\(_\text{s}\) elevation protects cells from PS-341–induced apoptosis.

**Discussion**

*Induction of DR4 and/or DR5 and enhancement of TRAIL-induced apoptosis by PS-341.* We have shown in this study that PS-341 increased DR4 and DR5 expression in NSCLC cells, including H157 and H460, which have wild-type and mutant p53 (H157 and H1792), respectively. These observations are consistent with previous reports that PS-341 induces DR4 and DR5 expression in primary chronic lymphocytic leukemia cells (37, 38). Moreover, the JNK inhibitor SP600125 only weakly attenuated PS-341–induced DR5 induction, suggesting that other mechanism(s) beyond JNK may be involved in PS-341–induced DR5 expression in human NSCLC cells. Because PS-341 induces endoplasmic reticulum stress, including up-regulation of CHOP/GADD153 (43, 44), it remains to be determined whether PS-341 induces apoptosis independently of TRAIL ligand in human NSCLC cells.

DR5 expression is regulated through p53-dependent and p53-independent mechanisms (2, 39). Although PS-341 was reported to increase p53 expression (20, 40), we found that PS-341 increased DR5 expression in NSCLC cell lines with wild-type p53 (H460) and mutant p53 (H157 and H1792). Thus, PS-341 is likely to up-regulate DR5 expression through a p53-independent mechanism. Some studies have shown that JNK regulates DR5 expression (6, 41, 42). Although PS-341 indeed induced JNK activation in our cell lines as shown previously (20, 21), we found that the JNK inhibitor SP600125 only weakly attenuated PS-341–induced DR5 induction, suggesting that other mechanism(s) beyond JNK may be involved in PS-341–induced DR5 expression in human NSCLC cells. Because PS-341 induces endoplasmic reticulum stress, including up-regulation of CHOP/GADD153 (43, 44), a transcriptional factor known to regulate DR5 expression (27, 45), it remains to be determined whether PS-341 induces a CHOP-dependent up-regulation of DR5.

**c-FLIP is regulated by a ubiquitin-proteasome mechanism.** c-FLIP expression is induced by a ubiquitin-proteasome system (46, 47), and certain cancer therapeutic agents stimulate down-regulation of c-FLIP expression through this mechanism (46). PS-341, as a proteasome inhibitor, was surprisingly reported to

\[^1\] Unpublished data.
reduce c-FLIP levels (24), although other studies showed that PS-341 did not alter c-FLIP levels (38) or increased the levels of c-FLIP in the DISC (48). In our study, we clearly showed that PS-341 increased the levels of FLIP<sub>L</sub> without altering FLIP<sub>S</sub> levels in all of the tested NSCLC cell lines. Moreover, we also showed that other proteasome inhibitors, including MG132 and epoxomicin, exhibited similar effects on modulation of c-FLIP expression as PS-341. Therefore, this is the first study to show that PS-341 and other proteasome inhibitors selectively increase FLIP<sub>L</sub> levels, although the underlying mechanism is currently unclear. A recent study has shown that FLIP<sub>S</sub> is more prone to ubiquitination and has a considerably shorter half-life in comparison with FLIP<sub>L</sub> (47). Therefore, it would be interesting to investigate whether PS-341 increases FLIP<sub>L</sub> levels through inhibition of the proteasome. Nevertheless, our results indicate that it is unlikely for PS-341 to enhance TRAIL-induced apoptosis through modulation of c-FLIP in human NSCLC cells.

The basal levels of c-FLIP in H157 and H460 cells, which were less sensitive to PS-341–induced apoptosis, were much higher than in H1792 cells, which were more sensitive to PS-341–induced apoptosis. Moreover, FLIP<sub>L</sub> levels were greatly increased in H157 and H460 cells in comparison with those in H1792 cells (Fig. 3). Thus, it seems that the levels of c-FLIP, particularly FLIP<sub>S</sub>, elevation during PS-341 treatment, may provide a protective mechanism for cells to counteract PS-341–induced apoptosis. Indeed, this notion was supported by our findings that reduction of c-FLIP levels, particularly prevention of c-FLIP<sub>S</sub> elevation during PS-341 treatment, may provide a protective mechanism for cells to counteract PS-341–induced apoptosis (Fig. 6).

Survivin, Bcl-2, and Bcl-X<sub>L</sub> are known to regulate apoptosis or enhance TRAIL-induced apoptosis through modulation of c-FLIP and survivin, PS-341 indeed induced apoptosis and enhanced TRAIL-induced apoptosis in human NSCLC cells (Fig. 3A). To the best of our knowledge, this is the first study showing that PS-341 increases survivin expression. Given the antiapoptotic property of survivin, it is likely that survivin up-regulation, like FLIP<sub>S</sub> elevation, may counteract the apoptosis-inducing effect of PS-341. It has been shown that the ubiquitin-proteasome regulates survivin degradation (49). Given that other proteasome inhibitors (e.g., MG132 and epoxomicin) other than PS-341 also increased survivin levels (Fig. 3C), it is plausible to speculate that PS-341 may stabilize survivin through inhibition of the proteasome. Studies to evaluate this hypothesis are ongoing.

Our results clearly indicate that PS-341 treatment generates conflicting signals by activating both proapoptotic (e.g., DR5) and antiapoptotic signaling (e.g., c-FLIP and survivin). Despite up-regulation of c-FLIP and survivin, PS-341 indeed induced apoptosis and enhanced TRAIL-induced apoptosis in human NSCLC cells albeit with various degrees. Thus, it seems that PS-341–activated proapoptotic signaling, such as DR5 induction, can override activation of antiapoptotic signaling, such as up-regulation of FLIP<sub>S</sub> and survivin caused by PS-341, leading to induction of apoptosis and enhancement of TRAIL-induced apoptosis. Given that prevention of c-FLIP elevation during PS-341 treatment sensitized cells to PS-341–induced apoptosis, it may be possible to enhance the anticancer efficacy of PS-341 via combination with other agents, which decrease c-FLIP and survivin expression.

It is known that TRAIL functions as a DR5 ligand and rapidly induces apoptosis in a wide variety of transformed cells but is not cytotoxic in normal cells in vitro and in vivo (1, 2). Therefore, TRAIL is considered to be a tumor-selective, apoptosis-inducing cytokine and a promising new candidate for cancer treatment. Unfortunately, certain cancer cell lines and tumors are resistant to TRAIL-mediated cell killing (2). In addition, agonistic anti-DR5 antibodies can also induce DR5 trimerization, which triggers the extrinsic apoptotic pathway, thus having great cancer therapeutic potential (50). In fact, the agonistic anti-DR5 antibody is already being tested in phase I clinical trials. Therefore, PS-341 may be useful in combination with TRAIL or an agonistic anti-DR5

Figure 6. Silencing of c-FLIP sensitizes NSCLC cells to PS-341 treatment. H460 and H157 cells seeded in a 96-well plate or a 24-well plate were transfected with control or c-FLIP siRNA. Twenty-four hours after the transfection, cells were treated with 50 nmol/L PS-341 (in a 24-well plate) for 24 h (A and C) or with the given concentrations of PS-341 for 48 h (B). The cells were then harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis to evaluate the silencing efficiency of c-FLIP (A) or subjected to the SRB assay for estimation of cell survival (B) or sub-G1 analysis for measurement of apoptosis (C). Columns, mean of triplicate treatments (B); bars, SD.
antibody to achieve an enhanced effect on apoptosis induction or overcome TRAIL resistance in human cancer cells.

In summary, our study has shown that PS-341 induces DR5 expression, which contributes to PS-341-induced apoptosis and enhancement of TRAIL-induced apoptosis in human NSCLC cells despite up-regulation of FLIPs and survivin. Our findings provide novel insight into the mechanism by which PS-341 induces apoptosis and enhances TRAIL-induced apoptosis in human cancer cells.

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