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Proteasome Inhibitor PS-341 (Bortezomib)Induces Calpain-dependent IκBα Degradation

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The proteasome, a key component of the ubiquitin-proteasome pathway, has emerged as an important cancer therapeutic target. PS-341 (also called Bortezomib or Velcade) is the first proteasome inhibitor approved for newly diagnosed and relapsed multiple myeloma and is currently being tested in many clinical trials against other types of cancers. One proposed mechanism by which PS-341 exerts its anticancer effect is inactivation of nuclear factor-κB (NF-κB) through prevention of IκBα degradation. In this study, we show that PS-341 at concentrations that effectively inhibited the growth of human cancer cells, instead of increasing IκBα stability, paradoxically induced IκBα degradation. As a result, PS-341 facilitated p65 nuclear translocation and increased NF-κB activity. Moreover, IκBα degradation by PS-341 occurred early before induction of apoptosis and could not be inhibited by a pan-caspase inhibitor or caspase-8 silencing; however, it could be prevented with calpain inhibitors, calcium-chelating agents, calpain knockdown, or calpastatin overexpression. In agreement, PS-341 increased calpain activity. These data together indicate that PS-341 induces a calpain-mediated IκBα degradation independent of caspases. In the presence of a calpain inhibitor, the apoptosis-inducing activity of PS-341 was dramatically enhanced. Collectively, these unexpected findings suggest not only a novel paradigm regarding the relationship between proteasome inhibition and NF-κB activity but also a strategy to enhance the anticancer efficacy of PS-341.

The proteasome, a key component of the ubiquitin-proteasome pathway, has emerged as an important cancer therapeutic target (1–3). PS-341 (also called Bortezomib or Velcade), a dipeptidyl boronic acid, is a highly specific proteasome inhibitor with potent anticancer activity (4, 5). It is an approved drug for treatment of patients with multiple myeloma and mantle cell lymphoma. Many preclinical studies have demonstrated that PS-341 alone or in combination with other cancer therapeutic agents 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. Currently, there are many ongoing clinical trials that test the anticancer efficacy of PS-341 or its combinations with other agents in several types of cancers, including lung cancer (2, 6, 7).

Nuclear factor-κB (NF-κB)5 is a transcription factor that regulates the expression of a large number of genes involved in multiple biological processes, including apoptosis (8, 9). Its activation is often associated with cell resistance to apoptosis via up-regulation of the expression of multiple anti-apoptotic genes such as Bcl-2 and c-FLIP (9, 10). Therefore, NF-κB has long been an attractive target for cancer therapy. It is well known that IκBα ubiquitination and its subsequent degradation by the 26S proteasome leading to nuclear translocation of p65 and an increase in NF-κB DNA binding are the primary mechanisms of NF-κB activation in cells upon stimulation (9, 10). Thus, a putative mechanism by which PS-341 induces apoptosis and exerts its anticancer activity is to inhibit NF-κB activation via prevention of IκBα degradation (1, 5).

PS-341 either as a single drug or in combination with other agents has been demonstrated to be active in inducing growth arrest and apoptosis in lung cancer cells in vitro and in inhibiting the growth of lung tumors in animal models (6, 7). To date, PS-341 is the only proteasome inhibitor to be evaluated in clinical trials against lung cancer and to show activity when used both as a single agent and in combination regimens (7). However, the mechanisms by which PS-341 induces apoptosis or growth arrest in lung cancer, particularly the role of NF-κB in these events, remain unclear.

Therefore, we have been interested in studying how PS-341 induces apoptosis in human cancer cells, particularly lung cancer cells. This study particularly focused on the role of NF-κB inhibition in PS-341-induced apoptosis in human lung cancer cells. Unexpectedly, we found that PS-341, instead of preventing IκBα degradation, induces calpain-dependent IκBα degradation in human lung cancer cells and other types of cancer cells. Moreover, we demonstrate that PS-341-induced IκBα degradation results in NF-κB activation and impacts cell responses to PS-341-induced apoptosis. Thus, this study sug-

5 The abbreviations used are: NF-κB, nuclear factor-κB; siRNA, small interfering RNA; IKK, IκB kinase; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid; Z, benzoylcarboxyl; FMK, fluoromethyl ketone; EST, [l-trans-3-ethoxy carbonylloxirane-2-carbonyl]-L-Leu-(3-methylbutyl)amide; PARP, poly(ADP-ribose) polymerase.
suggests a novel mechanism underlying PS-341-mediated biologi-
cal functions and a novel strategy to enhance the anticancer
efficacy of PS-341.

EXPERIMENTAL PROCEDURES

Reagents—PS-341 was provided by Millennium Pharmaceu-
ticals (Cambridge, MA). It was dissolved in dimethyl sulfoxide
(DMSO) at a concentration of 10 mM, and aliquots were stored
at −80 °C. Stock solutions were diluted to the desired final con-
centrations with growth medium just before use. The pan-
caspase inhibitor benzylacylcarbonyl-Val-Ala-Asp-fluoro-
methyl ketone (Z-VAD-FMK) was purchased from Enzyme
System Products (Livermore, CA). Ac-Leu-Leu-Val-Leu-7-amino-4-
methylcoumarin, and 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra-
aacetic acid (BAPTA) were purchased from Biomedical (Plymouth
Meeting, PA). Epoxomicin, [1-trans-3-ethoxyacylcarbonyloxirane-
2-carbonyl]-1-Leu-(3-methylbutyl)amide (EST), leupeptin, and
PD150606 were purchased from Calbiochem. MG132, EGTA,
and cisplatin were purchased from Sigma. The membrane-per-
meable calpain-specific fluorogenic substrate, t-butoxycarbo-
yl-Val-Leu-Met-chloromethylcoumarin, and calpastatin ex-
pression plasmid (3878564) were purchased from Invitrogen.
Docetaxel was obtained from Sanofi Aventis (Bridgewater, NJ).

Cell Lines and Cell Culture—All cell lines used in this study
were originally purchased from the American Type Culture
Collection (ATCC; Manassas, VA) and kept in our laboratory.
They were grown in monolayer culture in RPMI 1640 medium
supplemented with 5% fetal bovine serum at 37 °C in a humid-
ified atmosphere consisting of 5% CO₂ and 95% air.

Western Blot Analysis—The procedures for preparation of
whole-cell protein lysates and Western blotting were described
previously (11). Rabbit polyclonal antibodies against caspase-8,
caspase-9, calpastatin, and PARP and mouse monoclonal phos-
pho-IκBαSer-32/36) antibody were purchased from Cell Sig-
aling Technology, Inc. (Beverly, MA). Mouse monoclonal anti-
caspase-3 antibody was purchased from Imgenex (San
Diego). Mouse monoclonal anti-IκBα and anti-calpain regula-
tory subunit antibodies were purchased from Santa Cruz
Biotechnology. Rabbit polyclonal anti-actin and anti-gly-
ceraldehyde-3-phosphate dehydrogenase antibodies were
purchased from Sigma and Trevigen, Inc. (Gaithersburg,
MD), respectively.

Gene Silencing with Small Interfering RNA—The nonsilenc-
ing control and caspase-8 small interfering RNA duplexes
were described previously (12). Calpain regulatory subunit (SC-
29887) and calpastatin (SC-29889) siRNAs were purchased
from Santa Cruz Biotechnology. Transfection of these siRNA
duplexes was conducted in 6-mm dishes using the HiPerFect
transfection reagent (Qiagen, Valencia, CA) following the manu-
facturer’s fast forward protocol. Briefly, 1 × 10⁶ cells were
seeded into a 60-mm culture dish 1 h prior to transfection.
The given siRNA at 20 nM was mixed with 40 μl of HiPerFect
transfection reagent, diluted with Opti-MEM (Invitrogen)
medium, and then added to the cells. After 38 h post-transfection, the
cells were treated with 50 nM PS-341 for an additional 10 h
before collecting the cells for Western blotting. Gene silencing
effects and other protein expression were evaluated by Western
blot analysis as described above.

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Cell Survival Assay—Cells were seeded in 96-well cell culture
plates and treated the next day with the agents indicated. The
viable cell number was determined using the sulforhodamine B
assay, as described previously (13).

Detection of Apoptosis—Apoptosis was evaluated by annexin
V staining using annexin V-PE apoptosis detection kit pur-
bred from BD Biosciences following the manufacturer’s
instructions.

Proteasome Activity Assay—Fluorometric measurement of
20 S proteasome activity was the same as we described previ-
ously (14).

Calpain Activity Assay—Intracellular calpain activity was
measured as described previously (15, 16). Cells in a 96-well
plate or in chamber slides were exposed to PS-341 for a given
time and then loaded with 10 μM t-butoxycarbonyl-Leu-Met-
chloromethylcoumarin. After a 30–60-min incubation, cellu-
lar fluorescence was then quantified with a SPECTRAmax dual-
scanning microplate spectrofluorometer (Molecular Devices
Co., Sunnyvale, CA) with 351-nm excitation and 430-nm emis-
sion filters or recorded under a fluorescent microscope.

Indirect Immunofluorescent Staining—The procedure for
p65 staining using rabbit anti-human Rel/p65 antibody (Santa
Cruz Biotechnology, Inc.) was described previously (17).

Transient Transfection and Reporter Activity Assay—The
pNF-κB-luc reporter plasmid, which contains the luciferase
reporter gene driven by a basic promoter element (TATA box)
joined to seven repeats of an NF-κB-response element, was pur-
chased from Stratagene (La Jolla, CA). A pCH110 plasmid
encoding β-galactosidase was purchased from GE Healthcare
and used for normalization. The procedures for transfection,
luciferase activity assay, and β-galactosidase assay were
described previously (18).

Adenoviral Infection—Adenovirus harboring an empty vec-
ector (Ad-CMV) or a super repressor of IκBα (Ad-SRiκBα) was
generously provided by Dr. L. W. Chung (Emory University,
Atlanta, GA). The amplification, purification, and infection
of adenovirus were described previously (17).

RESULTS

PS-341 Reduces IκBα Levels by Induction of Its Proteolysis
while Inhibiting Proteasome Activity—IκBα is known to be
degraded via a ubiquitin-proteasome mechanism (9). PS-341 as
a proteasome inhibitor theoretically should stabilize IκBα pro-
tein through inhibition of proteasome activity. When examin-
ing the effects of PS-341 on IκBα degradation in two human
lung cancer cell lines, we surprisingly found that PS-341 de-
creased IκBα levels while increasing p-IκBα levels in a time-
dependent fashion. In H157 cells, PS-341 substantially
increased p-IκBα levels at 1 h followed by a great decrease in
IκBα levels after 2 h. In H460 cells, a drastic p-IκBα increase
was accompanied by a substantial reduction of IκBα levels after
a 2-h treatment. In both cell lines, the IκBα reduction was sus-
stained up to 12 h (Fig. 1A). By measuring the 20 S proteasome
activity, we found that PS-341, under the same experimental
conditions, effectively inhibited the 20 S proteasome activity
starting from 0.5 h post-treatment in both tested cell lines (Fig.
1B). Moreover, we examined the effects of different concentra-
tions of PS-341 on modulation of IκBα levels. As presented in
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Fig. 1C, PS-341 at all the tested concentrations (i.e. 25–1000 nm) decreased IκBα levels in both cell lines albeit without an apparent dose-dependent relationship in H157 cells. Accordingly, we detected increased levels of p-IκBα in both cell lines. In the other two lung cancer cell lines, PS-341 also effectively reduced the levels of IκBα (Fig. 1D). Moreover, we examined the effects of PS-341 on IκBα degradation in other types of solid tumor cell lines, including prostate (DU145), cervical (HeLa), breast (MCF-7), and head and neck cancer (SqCC/Y1), to determine whether IκBα reduction by PS-341 is a common phenomenon. In agreement with the findings in lung cancer cell lines, PS-341 at both low (50 nm) and high (1000 nm) concentrations decreased IκBα levels while increasing p-IκBα levels in all of the tested cell lines (Fig. 2A). Similar results were also obtained in several myeloma (U226, B226, and RPM/8226) cell lines (Fig. 2B).

Furthermore, we determined whether other proteasome inhibitors exerted similar effects as PS-341 in modulation of IκBα levels. We used three proteasome inhibitors MG132, epoxomicin, and ALLN in this experiment and found that all these agents increased p-IκBα levels and decreased IκBα levels in both H157 and H460 cells (Fig. 2C). Thus, it appears that reduction of IκBα levels is a common phenomenon for proteasome inhibitors.

We noted that a short form of a protein band could be detected in the Western blot analysis for IκBα from cells exposed to either PS-341 (Fig. 1C and Fig. 2, A and B) or other proteasome inhibitors (Fig. 2C). This short form of protein appeared or was increased as the IκBα levels were decreased. Given that IκBα can be cleaved by a calpain-dependent mechanism (19, 20), it is very likely that this short form of protein is a cleaved form of IκBα. Thus, it appears that inhibition of proteasome with either PS-341 or other inhibitors reduced IκBα through promotion of its cleavage.

We also examined the effects of cisplatin and docetaxel, two cancer chemotherapeutic agents, on IκBα levels in our cell systems in comparison with PS-341. Under the tested conditions, we found that only PS-341, but not cisplatin and docetaxel, increased p-IκBα levels and decreased IκBα levels with increased levels of cleaved fragment (supplemental Fig. S1), suggesting that IκBα degradation is unique to PS-341 treatment.

PS-341 Induces Calpain-dependent IκBα Proteolysis Independent of Caspases—IκBα has been shown to be degraded through proteasome-independent mechanisms (e.g. caspase or calpain protease) (19–22). In our study, we noted that cleavage of PARP, a hallmark of apoptosis, was detected only at 8 h after PS-341 treatment, which was far later than IκBα reduction (after 1 h) (Fig. 1A). Moreover, strong cleavage of IκBα was detected in DU145 and MCF-7 cells, in which PARP cleavage was minimally detected or not detected, whereas relatively weak IκBα cleavage was detected in 293FT, HeLa, and SqCC/Y1 cells in which PARP was strongly cleaved (Fig. 2A). These results together suggest that the observed IκBα cleavage is unlikely due to caspase activation. Indeed, in the presence of the pan-caspase inhibitor, Z-VAD-FMK, which at the tested concentration abolished PS-341-induced caspase-3 activation (supplemental Fig. S2), PS-341 still effectively induced IκBα reduction in both H157 and H460 cells (Fig. 3A). A previous study suggested that caspase-8 is involved in IκBα cleavage (23). Thus, we also specifically addressed whether caspase-8 is involved in mediating PS-341-induced IκBα degradation through inactivating caspase-8 by knocking down caspase-8 expression. As presented in Fig. 3A (lower panel), knockdown of caspase-8 failed to prevent PS-341-induced IκBα reduction, indicating that caspase 8 is not involved in IκBα degradation induced by PS-341. Collectively, these data strongly suggest that PS-341 induces a caspase-independent IκBα proteolysis.

In addition, we determined whether the trypsin-like protease and cysteine protease inhibitor, leupeptin, could prevent PS-341-induced IκBα degradation. As presented in Fig. 3A, PS-341-induced IκBα reduction could not be prevented by leu-
peptin in either H157 or H460 cells, indicating that PS-341 decreases IκBα levels independent of leupeptin-sensitive proteases as well.

Finally, we investigated whether PS-341 induces IκBα degradation through a calpain-mediated mechanism. To this end, we first examined whether the presence of calpain inhibitors abolished the ability of PS-341 to induce IκBα degradation. As presented in Fig. 3B, the presence of either EST or PD150506 prevented IκBα from reduction induced by PS-341 in both H157 and H460 cells. In agreement, another calpain inhibitor calpeptin inhibited PS-341-induced IκBα reduction in H460 cells as well. Furthermore, we silenced the expression of the calpain regulatory subunit, which is a common regulatory subunit for both μ-calpain (calpain 1) and m-calpain (calpain 2) (24), and we examined its effect on PS-341-induced IκBα degradation.

We assumed that knockdown of the calpain regulatory subunit would inhibit both μ-calpain and m-calpain activity. In agreement with the effects of the calpain inhibitors, as discussed above, knockdown of calpain regulatory subunit abolished the ability of PS-341 to reduce IκBα levels (Fig. 3C). These results clearly indicate that PS-341 induces calpain-dependent IκBα proteolysis.

It is known that calpains are calcium-dependent cysteine proteases (24). Thus, we further looked at the impact of calcium depletion on PS-341-induced IκBα degradation. The presence of the calcium-chelating agent BAPTA or EGTA alone in part prevented IκBα reduction by PS-341; however, the co-presence of both EGTA and BAPTA abolished the ability of PS-341 to decrease IκBα levels (Fig. 3D). These results again support the critical role of calpains in mediating PS-341-induced IκBα degradation.

Moreover, we determined whether PS-341 indeed increases calpain activity. As presented in Fig. 4, A and B, PS-341 significantly increased the proteolytic hydrolysis of the calpain-specific fluorogenic substrate, t-butoxycarbonyl-Leu-Met-chloromethylcoumarin, exhibiting increased fluorescence. These results indicate that PS-341 increases intracellular calpain activity. In the presence of the calpain inhibitor PD150606, PS-341 did not increase calpain activity (i.e. fluorescence intensity) (Fig. 4C), indicating the specificity of the assay for calpain activity. This result provides another piece of evidence supporting involvement of calpain in PS-341-induced IκBα degradation.

PS-341 induces IκBα proteolysis involving down-regulation of calpastatin—To gain insight into the mechanism underlying proteasome inhibition-induced calpain activation, we further examined whether PS-341 modulates the levels of calpastatin, a well known endogenous calpain inhibitor (25), because proteasome inhibition was reported to reduce calpastatin levels (26). In Western blotting, we detected multiple sizes (e.g. ~110, 70, and 40 kDa) of calpastatin, which were identical to published observations (27, 28). siRNA-mediated silencing of calpastatin substantially reduced the levels of these proteins, indicating that they are all specific calpastatin proteins. Meanwhile, IκBα levels were drastically reduced in calpastatin-silenced cells (Fig. 5A). PS-341 treatment decreased the levels of calpastatin accompanied by an increase in p-IκBα levels and reduction of IκBα in a time-dependent manner (Fig. 5B). Enforced expression of ectopic calpastatin increased basal levels of IκBα and prevented IκBα from degradation by PS-341 (Fig. 5C). Collectively, these results indicate that calpastatin inhibition is involved in mediating IκBα proteolysis by PS-341.

IκBα phosphorylation is required for PS-341-induced IκBα degradation—Given that inhibition of the proteasome with different proteasome inhibitors increases p-IκBα levels, we then asked whether IκBα phosphorylation is required for proteasome-inhibition-initiated IκBα degradation. To address this issue, we took advantage of SR-IκBα, which carries serine-to-alanine mutations at residues 32 and 36 and thus cannot be phosphorylated at Ser-32 and Ser-36. We first infected two lung cancer cell lines H460 and H157 with Ad-SR-IκBα and then treated cells with either PS-341 or MG132 to determine
Moreover, we examined NF-κB detected in PS-341-treated H460 or H157 cells, indicating that detected p65 staining in the cytoplasm in DMSO-treated H460 immunofluorescent staining. As shown in Fig. 6 we analyzed p65 localization in cells treated with PS-341 using NF-κB essential step for p65 nuclear translocation and subsequent Western blot analysis. The cells were then subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis for p65 levels. The levels of SRI-B-regulated genes were measured to determine the effect of PS-341 on increasing the levels of these proteins; however, the effects of PS-341 on increasing the levels of these proteins were abolished or attenuated in cells infected with Ad-SRIkBα (supplemental Fig. S3). These results clearly indicate that PS-341 modulates the expression of these proteins through an IκBα degradation- or NF-κB activation-dependent mechanism. Taken together with the above findings, we conclude that PS-341 increases NF-κB activity.

Moreover, we further analyzed the effects of PS-341 on p65 nuclear translocation in more cancer cell lines. Consistently, PS-341 induces p65 nuclear translocation in every tested cell line (supplemental Fig. S4). These data again support the notion that PS-341 activates NF-κB.

**Inhibition of Calpain Augments the Effect of PS-341 on Induction of Apoptosis**—Activation of NF-κB is generally associated with apoptosis resistance, although it can be pro-apoptotic under certain conditions (8, 9, 22, 31, 32). Therefore, we investigated the impact of IκBα degradation on PS-341-induced apoptosis. To do this, we compared the effects of PS-341 on cell survival and apoptosis in the absence and presence of a calpain inhibitor. As presented in Fig. 7A, the calpain inhibitor PD150606 itself had a very weak effect on decreasing cell numbers. The combination of PS-341 with PD150606 was much more potent than PS-341 alone in decreasing cell numbers in both tested cell lines. Consistently, the combination of PS-341 with PD150606 caused the most apoptotic cells compared with PS-341 alone in both cell lines (Fig. 7B). For example, in H460 cells, PS-341 alone induced 13% apoptosis; however, the combination of PS-341 and PD150606 caused more than 40% apoptosis (Fig. 7B). Thus, it is clear that the presence of PD150606 substantially enhances the effect of PS-341 on induction of apoptosis, implying that inhibition of calpain activation may augment the anticancer activity of PS-341.

**DISCUSSION**

Although inhibition of IκBα degradation and NF-κB activation is a putative mechanism for action of PS-341, no study has...
been done to demonstrate this mechanism in lung cancer cells. Our efforts in this regard have generated surprising results that instead of inhibiting IκBα degradation PS-341 actually potently decreases IκBα levels and results in NF-κB activation. These findings are consistent with three previous studies demonstrating that PS-341 as well other proteasome inhibitors induce IκBα degradation and activate NF-κB in endometrial carcinoma cell lines (33), the HepG2 liver cancer cell line (23), and multiple myeloma cells (34). We also found that PS-341 decreased IκBα levels in other types of cancer cell lines, including myeloma, prostate, cervical, breast, and head and neck cancer cells. One previous study shows that PS-341 indeed increases IκBα levels in human glioblastoma multiforme cells (35). Nevertheless, it seems that induction of IκBα degradation by PS-341 is likely to be a common event in many types of cancer cells.

It is known that activated IκB kinase (IKK) complex phosphorylates IκBα on serines 32 and 36, leading to subsequent ubiquitination and proteasome-mediated degradation of IκBα. Our results clearly show that PS-341 induces IκBα phosphorylation on serines 32 and 36 accompanied with reduction of IκBα levels. Moreover, we found that PS-341 failed to induce degradation of the IκBα mutant, SR-IκBα, in which serines 32 and 36 are mutated to alanines; rather, it increased the levels of SR-IκBα (Fig. 6A). Thus, we conclude that PS-341 induces phosphorylation-dependent IκBα degradation. This finding is in agreement with previous work demonstrating that PS-341 and other proteasome inhibitors induce IKK-dependent IκBα phosphorylation and degradation in other types of cancer cell lines through detection of phosphorylated IKKs and inhibition of IKKs with small molecules or siRNA (33, 34, 36). We assume that PS-341 may induce an IKK-dependent IκBα phosphorylation and degradation in lung cancer cells as well.
Before this study, it was unclear why PS-341 as a proteasome inhibitor induces IκBα degradation. It is worth mentioning that other proteasome inhibitors such as MG132 were also shown to induce IκBα degradation in colon cancer (36) and endometrial carcinoma cells (33). Our own data demonstrate that the proteasome inhibitors MG132, epoxomycin, and ALLN increase IκBα phosphorylation and decrease IκBα levels in human lung cancer cells as well. Thus, it is likely that proteasome inhibition is associated with PS-341-induced IκBα phosphorylation and degradation.

IκBα can also be degraded by proteases (e.g., calpain) or caspases (21, 22) in addition to proteasome. A recent study shows that PS-341 induces IκBα degradation involving caspase-8 in HepG2 cells (23). Our study demonstrates that PS-341 induces IκBα degradation or proteolysis independent of caspase activation, including caspase-8 activation based on the following facts. First, IκBα phosphorylation and degradation apparently occurred much earlier (at 2 h) than PARP cleavage (at 8 h) (Fig. 1A). Second, strong IκBα cleavage occurred in the cell lines where PARP cleavage was not detected or only minimally detected (Fig. 2A). Third, the presence of the pan-caspase inhibitor Z-VAD-FMK failed to prevent PS-341-induced IκBα degradation (Fig. 3A). Finally, inactivation of caspase-8 by siRNA-mediated knockdown of caspase-8 did not inhibit PS-341-induced IκBα degradation (Fig. 3A, lower panel).

It has been documented that IκBα can undergo calpain-mediated proteolysis, which is parallel to the ubiquitination-proteasome mechanism (19). Calpain-mediated IκBα proteolysis generates an ~30-kDa cleaved IκBα intermediate in a previous report (19). In our study, we also detected an ~30-kDa cleaved band of IκBα in cells exposed to PS-341 or other proteasome inhibitors (Figs. 1 and 2). The presence of the calpain inhibitor EST, calpeptin, or PD150606 abolished the ability of PS-341 to induce IκBα degradation (Fig. 3B). In addition, knockdown of the calpain regulatory subunit with a calpain regulatory siRNA abolished the ability of PS-341 to decrease IκBα levels (Fig. 3C). Consistently, the calcium chelators EGTA and BAPTA inhibited PS-341-induced IκBα degradation as well (Fig. 3D) because calpains are calcium-dependent proteases. Moreover, we detected increased calpain activity in cells exposed to PS-341 (Fig. 4). Together, these results clearly indicate that PS-341 induces IκBα proteolysis through a calpain-dependent mechanism. Thus, it is likely that inhibition of the proteasome may favor calpain activation, leading to IκBα degradation.

It is well known that calpain activity is negatively regulated by its endogenous inhibitor calpastatin (25). It was previously shown that proteasome inhibition induced calpastatin degradation, leading to calpain activation and subsequent Tau protein degradation in neuroblastoma cells (26). Similarly, we found that knockdown of calpastatin substantially decreased IκBα levels, further supporting the notion that calpain can cause IκBα degradation. Importantly, PS-341 decreased the levels of calpastatin accompanied with IκBα degradation (Fig. 5B). Consistently, overexpression of an exogenous calpastatin elevated basal IκBα levels and prevented IκBα from degradation by PS-341 (Fig. 5C). Thus, it appears that proteasome inhibition down-regulates or inhibits calpastatin, leading to calpain activation and subsequent IκBα degradation.
Because NF-κB regulates the expression of multiple anti-apoptotic genes, its activation is often associated with apoptosis resistance (9, 10, 32). In agreement with the finding in endometrial carcinoma cells (33) and multiple myeloma cells (34), we have also shown that PS-341 induces p65 nuclear translocation and increases NF-κB activity (Fig. 5) in our cell systems. Moreover, PS-341 increased the expression of survivin, c-FLIP, and COX-2, all of which are NF-κB-regulated genes (9, 29, 30). Importantly, suppression of IκBα degradation by expressing SR-IκBα abrogated the increase of these proteins by PS-341 (supplemental Fig. S3), furthering the notion that PS-341 activates NF-κB in our cell systems. In endometrial carcinoma cells, NF-κB activation does not mediate cytotoxic effects of proteasome inhibitors because neither IKKα nor IKKβ small
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hairpin RNA protected cells from death induced by these inhibitors (33). In multiple myeloma cells, inhibition of IKB enhances PS-341-induced cytotoxicity (34). In human lung cancer cells, we found that the presence of a calpain inhibitor (e.g. PD150606), which inhibits PS-341-induced IκBα degradation, substantially enhanced the effects of PS-341 on decreasing cell survival and inducing apoptosis (Fig. 7), implying that IκBα degradation consequently confers cell resistance to undergo apoptosis, presumably through activation of NF-kB. Thus, our findings in this regard may suggest a novel therapeutic strategy for enhancing the anticancer activity of PS-341 by preventing calpain activation.

In summary, we report a novel finding that PS-341 induces calpain-dependent IκBα degradation in human lung cancer as well other types of cancer cells. As a result, PS-341 activates NF-κB, which can confer apoptotic resistance and counteract the apoptosis inducing activity of PS-341. Thus, prevention of calpain-dependent IκBα degradation will substantially augment the apoptosis inducing activity of PS-341.

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Fig. S1. Effects of PS-341, cisplatin and docetaxel on IκBα phosphorylation and degradation. H157 cells were treated with 50 nM PS-341, 5 μM cisplatin and 50 nM docetaxel for 8 h and then harvested for preparation of whole cell protein lysates and subsequent Western blot analysis. ΔIκBα, cleaved IκBα.
Fig. S2. Z-VAD-FMK blocks PS-341-induced increase in caspase-3 activity. The indicated cell lines were incubated with 50 nM PS-341 in the absence and presence of 50 μM Z-VAD-FMK for 24 h and then subjected to caspase-3 activity assay. Columns, means of four replicate determinations; Bars, ± SD.
**Fig. S3.** PS-341 increases survivin, c-FLIP and COX-2 through a NF-κB-dependent mechanism. H157 cells were infected with 50 MOI Ad-CMV or Ad-SR-IκBα for 48 h. The cells were treated with DMSO or 50 nM PS-341 for additional 8 h and then harvested for preparation of whole cell protein lysates and subsequent Western blot analysis.
Fig. S4. **PS-341 induces p65 nuclear translocation in human cancer cell lines.** The given cell lines were treated with DMSO or 50 nM PS-341 for 4 h and then subjected to immunofluorescent staining of p65.