

PS-341, a Novel Proteasome Inhibitor, Induces Bcl-2 Phosphorylation and Cleavage in Association with G₂-M Phase Arrest and Apoptosis¹

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Abstract

Treatment with the proteasome inhibitor, PS-341 resulted in concentration- and time-dependent effects on Bcl-2 phosphorylation and cleavage in H460 cells that coincided with the PS-341-induced G₂-M phase arrest. The observed Bcl-2 cleavage paralleled the degree of PS-341-induced apoptosis but was detected to a similar extent with comparable concentrations of two other proteasome inhibitors (MG-132 and PSI). Calpain inhibitors, ALLM and ALLN, and the caspase inhibitors, Z-VAD and AC-YVAD did not induce Bcl-2 phosphorylation and cleavage. Exposure to PS-341 resulted in an additional *M_r* 25,000 cleavage fragment of Bcl-2, whereas only a *M_r* 23,000 fragment was observed with other anticancer agents. The formation of the *M_r* 25,000 fragment was not prevented by caspase inhibitors unlike the *M_r* 23,000 fragment, which suggests mediation by a caspase-independent pathway. Cell fractionation studies revealed that the Bcl-2 cleaved fragments localize within membrane structures and was an early event (at ~12 h, posttreatment), and before the observed cleavage of poly(ADP-ribose) polymerase (PARP), β-catenin, and DNA fragmentation (at ~36 h posttreatment). The *M_r* 23,000 Bcl-2 cleavage product was inhibited by the pan-caspase inhibitor and the inhibitors of caspase-3, -8, -9; but the PARP cleavage was prevented only by the pan-caspase and caspase-3 inhibitors, which suggests that the *M_r* 23,000 Bcl-2 cleavage occurred at both the initiation and execution stages of apoptosis. The inhibition of the ubiquitin/proteasome pathway by PS-341 leads, at an early stage of apoptosis, to Bcl-2 phosphorylation and a unique proteolytic cleavage

product, which are associated with G₂-M phase arrest and the induction of apoptosis.

Introduction

Apoptosis or programmed cell death is a genetically regulated cellular suicide mechanism that plays a crucial role in organelle development and the defense of homeostasis (1–3). Recent apoptosis research has been focused on the cascade of signaling events that leads to cell death and the mechanisms that regulate such a process (4–6). *Bcl-2* was first identified as an antiapoptotic proto-oncogene in non-Hodgkin's follicular lymphoma cells (7), and is located at chromosome t(14;18). *Bcl-2* as a key regulator of apoptosis, promotes cell survival by inhibiting factors needed for the activation of the caspases (cysteine proteases with aspartate-specificity) that dismantle cells (8–10). *Bcl-2* and its related family of proteins serve as a critical decisional point upstream to the induction of irreversible cellular damage, where these proteins focus much of their activation at the level of mitochondria (11). The conformational structure of *Bcl-2* family proteins are converted from inactive to active forms by posttranslational modifications occurring in response to cell death or survival signaling (12–13). These modifications include phosphorylation, dimerization, and proteolytic cleavage and often lead to subcellular translocation (14). *Bcl-2* phosphorylation can be induced after treatment with a variety of cytotoxic molecules including antitubulin agents paclitaxel, *Vinca* alkaloids, and nocodazole; the phosphatase inhibitor, okadaic acid; the PKC³ inhibitor, bryostatin; and by interleukin-3 (15–19). However, the functional significance of the observed *Bcl-2* phosphorylation remains unclear. Although *Bcl-2* phosphorylation is related to the triggering of apoptosis, other work has demonstrated that *Bcl-2* phosphorylation is associated with arrest of the cell cycle at G₂-M phase (20–22). In some of types of apoptotic cell death, work by Cheng *et al.* (23) has demonstrated that cleavage of *Bcl-2* occurred at NH₂-terminal loop domains by caspases, and such proteolysis cleavage leads to bax-like pro-apoptotic activity.

The ubiquitin/proteasome system plays an important role in the degradation of intracellular dysfunctional proteins and the rapid turnover of key regulatory proteins including cell cycle regulators and gene transcription factors, as well as

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³ The abbreviations used are: PKC, protein kinase C; PARP, poly(ADP-ribose) polymerase; TBST, Tris-buffered saline with Tween 20; PBMC, peripheral blood mononuclear cell; Top, topoisomerase; PSI, *N*-benzyloxycarbonyl-Ile-Glu (O-*t*-butyl)-Ala-Leucinal; ALLN, *N*-acetyl-Leu-Leu-Nle-CHO; ALLM, *N*-acetyl-Leu-Leu-Met-CHO, Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Z-DEVD, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone; AC-YVAD-FMK, *N*-acetyl-Try-Val-Ala-Asp-fluoromethylketone.

oncogene proteins (24–26). In addition, this key cellular system is also implicated in the regulation of cell proliferation, differentiation, survival, and apoptosis (27–29). Interest in the ubiquitin/proteasome pathway has led to the development of a number of proteasome inhibitors that have potent activities against a variety of cancer cell lines (30–32). PS-341 is a novel dipeptide boronate proteasome inhibitor developed by Millennium Pharmaceuticals Inc. (33, 34). In other work, we found that PS-341 exhibits a 30- to 300-fold greater range of activity against H460 cell growth than that observed with the proteasome inhibitors, MG-132 and PSI. Furthermore, we found that PS-341 caused stabilization of p53 protein, induced p53-related gene expression, blocked the cell-cycle progression at G₂-M phase, and finally induced programmed cell death. The results presented in this study demonstrate that PS-341 treatment lead to Bcl-2 phosphorylation and identification of a new cleavage product in a concentration- and time-dependent manner. The Bcl-2 cleavage is mediated by caspases as an early event in apoptotic process. In addition, PS-341 treatment led to the identification of a unique cleavage fragment (*M*, 25,000), which is not mediated by caspases and localized at membrane structures. Our data implicate PS-341 is a non-caspase epitope. These findings help build the rationale for use as a single agent and in combination treatment with other types of anticancer agents in clinical trials.

Materials and Methods

Chemicals. PS-341 was supplied by Millennium Pharmaceuticals Inc. (Cambridge, MA). PS-341 was dissolved in DMSO (1 mM) as a stock solution and diluted to the required concentration with PBS. PSI [Z-IE(OtBu)AL-CHO] and caspase inhibitors, Z-VAD-FMK, Z-DEVD-FMK, and AC-YVAD-FMK were purchased from Bachem Bioscience Inc. (King of Prussia, PA). MG-132 (Z-LLL-CHO) and the Calpain inhibitors, ALLN and ALLM were purchased from Biomol, Inc. (Plymouth Meeting, PA). Monoclonal anti-Bcl-2 antibody was purchased from Calbiochem (Cambridge, MA), and monoclonal anti-PARP and β -catenin antibodies were purchased from PharMingen, Inc. (San Diego, CA). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. H460 cells were purchased from the American Type Culture Collection (Rockville, MD), and grown in RPMI 1640 supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO₂ and 95% air.

Cell Cycle and Apoptosis Assay. Cells were harvested, fixed with cold 75% ethanol at –20°C overnight and incubated with 1 μ g/ml of propidium iodide and 5 μ g/ml of RNase I (Roche Molecular Biochemicals, Indianapolis, IN) at room temperature for 3 h. The cell cycle distribution and apoptotic cells (sub-G₀-G₁) were determined by flow cytometry (Epics Profile Analyzer; Coulter Co., Miami, FL). For assay of mitotic cells, cells were stained with Wright-Giemsa dye solution, and the mitotic cells were manually counted (at least 100 cells) under microscopy. The DNA fragmentation was determined as described previously (35).

Western Blot Analysis. Cells were scraped from the culture, washed twice with PBS, and then suspended in 30 μ l of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl,

5 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 20 μ g/ml leupeptin, 20 μ g/ml aprotinin, 0.1% Triton X-100, and 1% SDS at 0°C to 4°C for 15 min. After centrifugation at 1500 \times *g* for 10 min at 0°C, the supernatants were collected, and protein amount was assessed by a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Equal amounts (50 μ g of protein) of lysate were subjected to a 15% SDS-PAGE. After electrophoresis, protein blots were transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in TBST solution, and incubated overnight with the corresponding primary antibodies in the blocking solution at 4°C. After washing three times with TBST solution, the membrane was incubated at room temperature for 1 h, with horseradish peroxidase-conjugated secondary antibody diluted with TBST solution (1:1,000). The signals of detected proteins were visualized by an enhanced chemiluminescence reaction (ECL) system (Amersham, Arlington Heights, IL).

Subcellular Fractions. Cells were treated with 0.1 μ M PS-341 or with the same volume of PBS as a control for 24 h. Cells were washed twice with cold PBS, and separated into Triton X-100-soluble and -insoluble fractions as described by Lampugnani *et al.* (36). Briefly, cells were extracted with lysis buffer containing 10 mM Tri-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 10 μ g/ml each leupeptin and aprotinin, 1% NP40, and 1% Triton X-100 for 30 min in ice bath with gentle agitation. After centrifugation at 14,000 \times *g* at 4°C for 10 min, supernatant was collected as Triton X-100-soluble fraction, and the pellets were extracted as described above with 1% SDS-containing buffer in an ice bath for 20 min. After centrifugation at 14,000 \times *g* at 4°C for 10 min, the supernatant was collected as a Triton X-100-insoluble fraction.

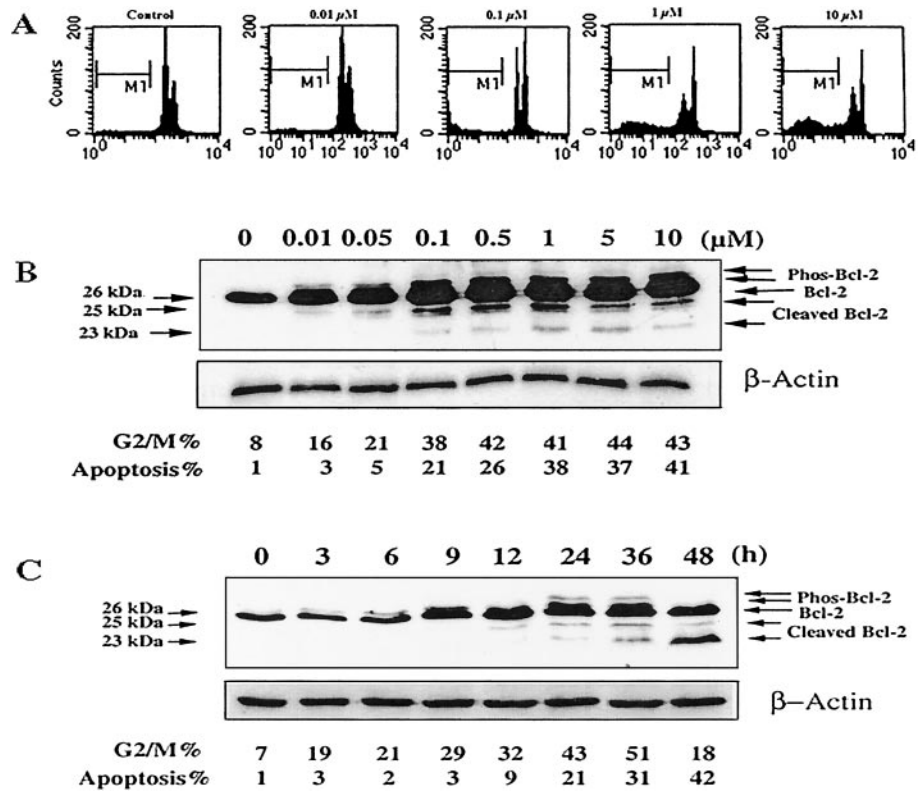
Mononuclear Cell Preparation. Blood samples were serially obtained from a patient enrolled at the 1.75-mg/m² dose level of a Phase I trial of PS-341 in advanced cancers (National Cancer Institute T99-047). Samples (7 ml) before PS-341 administration and at 1, 4, and 24 h after administration were collected in Becton Dickinson cell preparation tubes with sodium heparin and centrifuged at 1700 \times *g* for 20 min. The cell preparation tubes were inverted several times after centrifugation, and the mononuclear plasma preparation was removed and washed twice with 15-ml washes with PBS using 10-min 1000 \times *g* centrifugation steps. The cell pellet was then processed as described above for Western blot analysis with detection of Bcl-2.

Results

Effect of PS-341 on Bcl-2 Phosphorylation and Cleavage.

We had previously shown that PS-341 treatment caused the cell cycle arrest at G₂-M phase and programmed cell death in H460 cells (37). Therefore, it was of interest to determine whether this agent could induce Bcl-2 phosphorylation, and whether the resulting Bcl-2 phosphorylation could be associated with G₂-M phase arrest and/or the initiation of apoptosis. We treated H460 cells initially with different concentrations of PS-341 for a 24-h time period and then followed the effects of this treatment in terms of the percentage of cells at G₂-M and the percentage of apoptosis determined by

Fig. 1. PS-341-induced Bcl-2 phosphorylation and cleavage correlated with G₂-M phase arrest and apoptosis. H460 cells were exposed to varying concentrations of PS-341 for 24 h. After drug exposure, cell cycle distribution and apoptosis were determined by flow cytometry analysis as described in "Materials and Methods" (A). In addition, H460 cells were exposed to various concentrations of PS-341 for 24 h (B) or to 0.1 μ M PS-341 for the indicated times (C). After exposure, cells taken from culture were divided into two parts. One was for the determination of Bcl-2 phosphorylation and cleavage by Western blot analysis. Another was fixed with cold 75% ethanol and incubated with propidium iodide for the determination of cell cycle distribution and apoptosis by flow cytometry. Each data point represents the mean of two independent experiments. β -Actin was used as a sample loading control. *kDa*, molecular weight in thousands.



flow cytometry analysis, as well as the Bcl-2 phosphorylation pattern as detected by Western blot analysis. The results showed that PS-341 treatment caused cell accumulation at G₂-M phase and induced cell apoptotic death in a concentration-dependent manner (Fig. 1A). The Western blot analysis demonstrated that PS-341 treatment leads to a concentration-dependent phosphorylation of Bcl-2 protein that also parallels the increase of cells at G₂-M as well as the percentage of cells killed by apoptotic death (Fig. 1B). The slower mobility of the Bcl-2 band corresponded to the phosphorylation of the Bcl-2 protein and could be seen in the cells exposed to 0.01–0.05 μ M PS-341 for 24 h. Another slower band could be discerned, which corresponded to a superphosphorylated form of Bcl-2, and was detected when cells were exposed to higher concentrations of PS-341 (0.1–10 μ M) for 24 h. The extent of Bcl-2 phosphorylation increased proportionally with the PS-341 concentration. The exposure of H460 cells to 0.1 μ M PS-341 was followed with respect to the time dependence (0–48 h) of these effects on G₂-M arrest, apoptosis, and Bcl-2 cleavage (Fig. 1C). Starting at 12 h, discernable Bcl-2 cleavage products and an increase in the percentage of cells at G₂-M were seen. With respect to the time course of apoptosis, the key effects started at 24 h and progressed out to 48 h. The PS-341 treatment caused the cleavage of Bcl-2 protein into at least two fragments. The first cleavage fragment (*M_r* 25,000) of Bcl-2 protein was seen after a 24-h exposure to PS-341 at 0.01–0.05 μ M, or after a 12-h of exposure to 0.1 μ M. However, the second cleavage fragment (*M_r* 23,000) of the Bcl-2 protein was detected when cells were treated with 0.1 μ M PS-341 for 12 h. In addition,

Bcl-2 phosphorylation preceded Bcl-2 cleavage by at least 9 h. The extent of Bcl-2 phosphorylation was also coincident with the PS-341-induced G₂-M phase arrest. The proteolysis of Bcl-2 into a *M_r* 23,000 fragment also correlated with the PS-341-induced apoptosis.

Other protease inhibitors, as well as several calpain and caspase inhibitors were tested for similar effects with respect to the G₂-M phase arrest, Bcl-2 phosphorylation, Bcl-2 protein cleavage, and apoptosis. H460 cells were treated with equal cytotoxic concentrations of PS-341 (0.1 μ M), MG-132 (5 μ M), PSI (30 μ M), the calpain inhibitors, ALLN (20 μ M), and ALLM (20 μ M); and caspase inhibitors, Z-VAD-FMK (50 μ M) and AC-YVAD-FMK (50 μ M). After a 24-h incubation to each of these agents, cells were harvested from culture and divided into two parts. One part was incubated with propidium iodide for the assay of cell cycle distribution and degree of apoptosis. The other part was subjected to Western blot analysis. The treatment with the general proteasome inhibitor, MG-132, or with the chymotrypsin-like proteasome inhibitor, PSI, resulted in cell cycle arrest at G₂-M phase as well as apoptosis similar to the exposure to these proteasome inhibitors that also led to Bcl-2 protein phosphorylation and cleavage to an extent that was similar to that observed with PS-341. In contrast, treatment with calpain inhibitors, ALLN and ALLM, did not markedly alter cell cycle progression or lead to the induction of apoptosis. Both ALLN and ALLM did not result in any effects on the Bcl-2 phosphorylation and cleavage, but a significant increase in the amount of Bcl-2 protein content was observed. Treatment with the caspase inhibitors, Z-VAD-FMK and AC-YVAD-FMK showed no af-

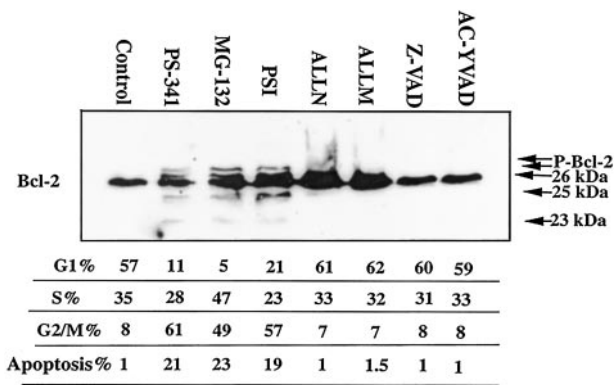


Fig. 2. Effects of different kinds of protease inhibitors on Bcl-2 phosphorylation and cleavage in H460 cells. Cells were treated with 0.1 μM PS-41, 5 μM MG-132, 30 μM PSI, 20 μM ALLN, 20 μM ALLM, 50 μM Z-VAD, or 50 μM AC-YVAD, and with the same volume of PBS solution as control. After 24 h of treatment, cells taken from culture were divided into two parts. One was for the determination of Bcl-2 phosphorylation and cleavage by Western blot analysis. Another was for the determination of cell cycle distribution and apoptosis as described in Fig. 1. Each data point represents the mean of two independent experiments. *kDa*, molecular weight in thousands.

ffects on the cell cycle progression, induction of apoptosis, or phosphorylation and cleavage of Bcl-2 protein (Fig. 2).

PS-341 Induces a Specific Cleavage Fragment of Bcl-2 Protein. Although some reports have indicated that Bcl-2 protein is cleaved into a M_r 23,000 fragment by several stimuli such as virus infection and treatment with etoposide and MG132 (38–40), we sought to examine the degree of specificity for the induction of the Bcl-2 additional cleavage fragment (M_r 25,000 product) with respect to effects with other types of anticancer agents at comparable cytotoxic concentrations. The treatment with PS-341 resulted in the induction of two cleavage fragment bands (Fig. 3), whereas treatment with anticancer agents including antitubulin agents, paclitaxel and vinblastine; the Top I inhibitor, camptothecin; the Top II inhibitor, etoposide; the anthracycline antibiotic, doxorubicin; and the PKC inhibitor, staurosporine, produced only a M_r 23,000 cleavage fragment. It was apparent that only proteasome inhibitors were able to produce this specific and unique Bcl-2 M_r 25,000 cleavage fragment.

Subcellular Localization of Phosphorylated and Cleaved Bcl-2. We next sought to determine whether the effects by PS-341 on the Bcl-2 phosphorylation and proteolytic cleavage could alter its subcellular distribution. H460 cells were subjected to treatment with 0.1 μM PS-341 and then separated into detergent-soluble and -insoluble fractions. The results showed that PS-341-induced phosphorylated Bcl-2 bands were observed in both Triton X-100 soluble and -insoluble fractions. The M_r 23,000 cleavage band was predominantly detected at the Triton X-100-insoluble fraction, and only a faint signal of this band was seen in Triton X-100-soluble fraction. The M_r 25,000 cleavage product was detected only at Triton X-100-insoluble fraction (Fig. 4).

Bcl-2 Cleavage Is an Early Event and Mediated by Initiator Caspases in PS-341-induced Apoptosis. The time course of the Bcl-2 cleavage was compared with that of DNA fragmentation and cleavage of caspase-3-like mediated-

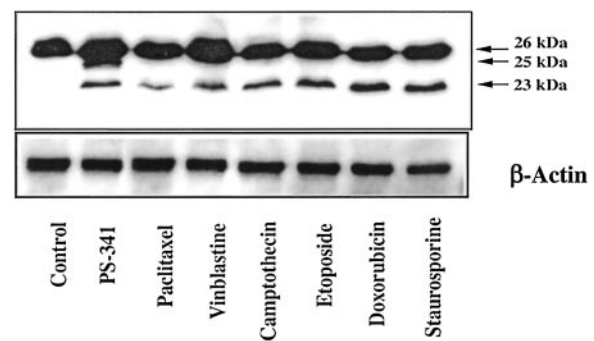


Fig. 3. Bcl-2 proteolytic cleavage by PS-341 and other different kinds of anticancer agents. H460 cells were treated with 0.1 μM PS-341, 0.5 μM paclitaxel, 0.1 μM vinblastine, 1 μM camptothecin, 5 μM etoposide, 0.25 μM doxorubicin, or 1 μM staurosporine, and with the same volume of PBS solution as control. After 48 h of incubation, cells were lysed with lysis buffer, and protein amount was assessed with a Bio-Rad DC protein assay kit as described in "Materials and Methods." Equal amounts (50 μg of protein) of lysate were subjected to a 15% SDS-PAGE. After electrophoresis, protein blots were transferred on a nitrocellulose membrane, and Bcl-2 protein was probed by a monoclonal anti-Bcl-2 antibody. β -Actin was used as a sample loading control. *kDa*, molecular weight in thousands.

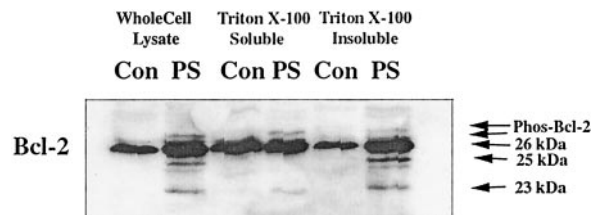


Fig. 4. Biochemical studies on subcellular distribution of phosphorylated and cleaved Bcl-2 protein in PS-341-treated H460 cells. Cells were treated with 0.1 μM PS-341 or with the same volume of PBS solution as control. After 24 h of incubation, cells taken from culture were extracted by lysis buffer containing Triton X-100. After centrifugation, the supernatant was collected as Triton X-100-soluble fraction. The pellet was dissolved in lysis buffer containing 1% SDS and collected as Triton X-100-insoluble fraction. All of the samples were subjected to a 15% SDS-PAGE. After electrophoresis, protein blots were transferred on a nitrocellulose membrane. The Bcl-2 protein was probed by monoclonal anti-Bcl-2 antibody. *kDa*, molecular weight in thousands.

target proteins PARP and β -catenin in H460 cells after treatment with 0.1 μM PS-341. The PS-341-induced DNA fragmentation was detected at about 36 h (Fig. 5A), and peaked at 48–72 h. These results correlated with the assessment of apoptotic cells by flow cytometry (Fig. 5B). Time course studies revealed that Bcl-2 cleavage occurred starting at 12 h with more discernable effects at 36–48 h after treatment, whereas the cleavage of PARP and β -catenin was observed at 36 h (Fig. 5C).

In a parallel fashion, we examined whether the cleavage of Bcl-2 could be used in an *in vivo* setting in surrogate PBMCs to monitor the clinical activity of PS-341 in ongoing and future Phase I and II clinical studies. Fig. 6 shows the Western blot analysis of peripheral mononuclear cells obtained from a patient enrolled in a Cancer Therapy Evaluation Program-sponsored Phase I study with PS-341 receiving treatment at the 1.75-mg/m² dose level. Serial blood samples

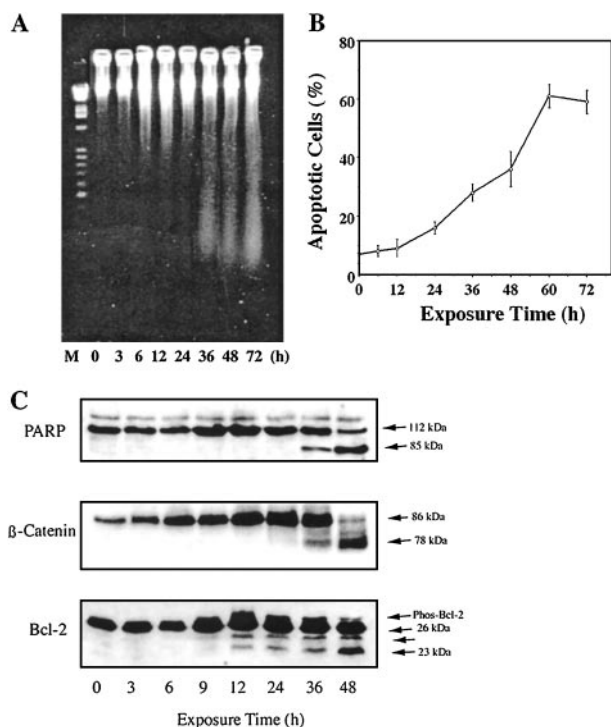


Fig. 5. Time course studies on PS-341-induced DNA fragmentation, apoptosis, and cleavage of PARP, β -catenin, and Bcl-2 protein. H460 cells were treated with $0.1 \mu\text{M}$ PS-341 for the indicated time. After treatment, cells taken from culture were divided into three parts. One was prepared for the determination of DNA fragmentation using agarose gel electrophoresis (A). The second part was prepared for the determination of apoptosis after cells stained with propidium iodide by flow cytometry (B). The third was prepared for the determination of cleavage of PARP, β -catenin, and Bcl-2 by Western blot analysis (C). Each point in B represents the mean \pm SD of three independent experiments. *kDa*, molecular weight in thousands.

were probed by Western blot analyses for Bcl-2 before (0) and 1, 4, and 24 h after the i.v. dosing of PS-341 on day 1 of the treatment cycle. At 1 h post PS-341 exposure the Bcl-2 phosphorylation product could be discerned. The presence of the M_r 25,000 Bcl-2 cleavage was evident at 4 h post-treatment along with the Bcl-2 phosphorylation product. At 24 h, we were able to observe a result parallel to the results observed with H460 cells, with the appearance of the additional M_r 23,000 cleavage product. This appeared as a consistent pattern after the 2 days of PS-341 dosing. It was also clear that there was a recovery from the effects of PS-341 treatment in the baseline PBMC sample before the day-4 treatment with PS-341, in agreement with 20S proteasome activity measurements performed on patient samples.

In other studies, we found that PS-341 exposure led to the activation of different types of caspase families at initiation and execution steps of apoptosis (37). Therefore, we sought to identify which caspases might be involved in PS-341-induced Bcl-2 cleavage. H460 cells were treated with $0.1 \mu\text{M}$ PS-341 alone or with PS-341 plus $50 \mu\text{M}$ of a number of different types of caspase inhibitors. The percentage of apoptotic cells and the cleavage of PARP and Bcl-2 protein after 48 h of treatment revealed that the pan-caspase inhib-

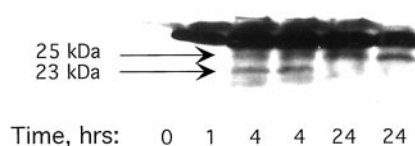


Fig. 6. Assessment of Bcl-2 cleavage in human PBMCs as a function of *in vivo* exposure to PS-341. As part of pharmacodynamic studies for the assessment of the degree of proteasome inhibition during a Phase I clinical study, mononuclear cells were probed by Western blot analysis using a 15% gel as carried out for H460 cells. A similar cleavage pattern of Bcl-2 to that detected with H460 cells can be discerned at the 4-h sampling, with a more pronounced effect occurring 24 h after the dosing of PS-341. This sequence of induction of Bcl-2 cleavage peptides was found to be repeated after a second dose of PS-341 on day 4 of a 2-week treatment cycle that involved dosing of PS-341 at days 1 and 4. *kDa*, molecular weight in thousands.

itor, Z-VAD, showed a remarkable inhibition of drug-induced apoptosis although the caspase-3 inhibitor, Z-DEVD, caspase-8 inhibitor, Ac-IETD, and caspase-9 inhibitors, Ac-LEHD, were also able to partially rescue the PS-341-induced apoptosis. (Fig. 7). However, the caspase-1 inhibitor, Ac-YVAD, showed no effect on drug-induced apoptosis. Although the pan-caspase inhibitor markedly prevented PARP cleavage, the caspase-3 inhibitor had a potent effect on the inhibition of PARP cleavage, whereas the caspase-1, -8, and -9 inhibitors had no effect on the prevention of PARP cleavage. All of the caspase inhibitors, with the exception of caspase-1 inhibitor, were capable of preventing PS-341-induced cleavage of Bcl-2 protein into M_r 23,000 fragment, but not of affecting the M_r 25,000 fragment.

Discussion

In other studies, we found that PS-341 is a highly potent proteasome inhibitor with strong activity against cell growth using the human non-small cell lung cancer H460 cell line. Cytotoxicity studies showed that PS-341 was about 30-fold to 300-fold more potent than the proteasome inhibitors, MG-132 and PSI, respectively and at low concentrations, caused cell cycle arrest at G_2 -M phase, which was shown to be via drug-induced blockade of p53 degradation and/or induction of p53-related gene expression along with the accumulation of cyclin B and cyclin A. None of these effects had any correlation with perturbations on tubulin polymerization and depolymerization (37). In the present studies, the PS-341-induced G_2 -M phase arrest was correlated to Bcl-2 phosphorylation, and the extent of the Bcl-2 phosphorylation was coincident with the accumulation of substantial cell numbers at G_2 -M phase, an activity similar to that of antitubulin agents such as paclitaxel and *Vinca* alkaloids. The Bcl-2 phosphorylation was not exclusively implicated with the disturbance of microtubule events related to G_2 -M phase arrest. Although several reports indicated that Bcl-2 phosphorylation could be mediated by several kinds of kinases including Raf1, protein kinase A, PKC, cdc-2, and c-Jun N-terminal Kinase, the actual kinase(s) that responds to Bcl-2 phosphorylation remains unclear (41, 42). Pharmacological concentrations of kinase inhibitors, including the PKC kinase inhibitor staurosporine, the tyrosine kinase inhibitor genistein, the cdc2 kinase inhibitor roscovitine, and the mitogen-activated protein

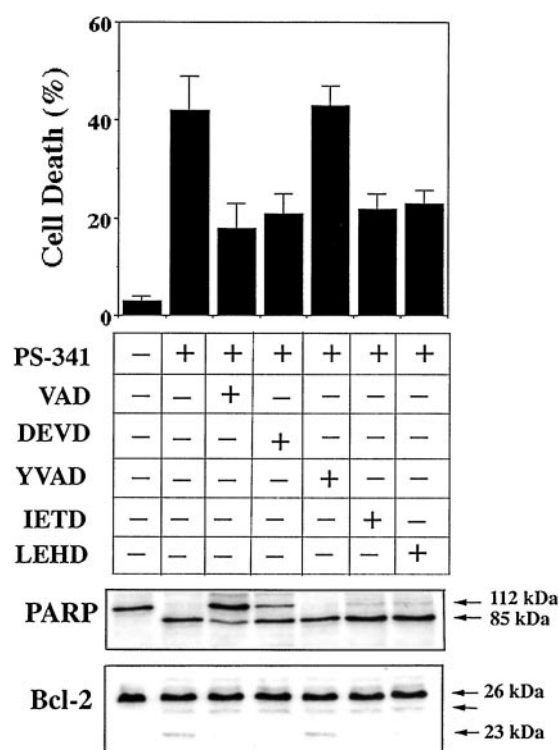


Fig. 7. Effects of different types of caspase inhibitors on PS-341-induced apoptosis and on the cleavage of PARP and Bcl-2. H460 cells were treated with 0.1 μM PS-341 alone, or with 50 μM z-VAD, Z-VEVD, AC-YVAD, AC-IETD, or AC-LEHD, and with the same volume of PBS solution as control. After 48 h of treatment, cells were harvested and divide into two parts. One was prepared for the determination of apoptosis as described in Fig. 5. (Top panel, each bar, the mean \pm SD of three independent experiments.) Another was prepared for the determination of cleavage of PARP and Bcl-2 by Western blot analysis as described in Fig. 5.

kinase inhibitor PD98059, were used to test which kinase-related pathway may be involved in PS-341-induced Bcl-2 phosphorylation. Preliminary data indicate that cotreatment with 0.1 μM PS-341 and 0.05 μM staurosporine results in a strong inhibition of PS-341-induced Bcl-2 phosphorylation. Cotreatment with 1 μM roscovitine only partially inhibited PS-341-induced Bcl-2 phosphorylation. In contrast, cotreatment with 5 μM genistein and 10 μM PD-98059 showed no effect on PS-341-induced Bcl-2 phosphorylation (data not shown). These results indicate that Bcl-2 phosphorylation by PS-341 might be mediated by PKC and cdc-2 kinase-related pathways.

Although the functional significance of Bcl-2 phosphorylation remains to be defined, some studies have shown that phosphorylation of Bcl-2 could result in a change in conformation and the loss of this protein's capability for membrane integration (43). In this study, we found that PS-341 treatment resulted in the phosphorylation and cleavage of Bcl-2. In addition, we found that Bcl-2 phosphorylation preceded the Bcl-2 cleavage by at least \sim 9 h. This observation raises a possibility that Bcl-2 phosphorylation could be a required event for the triggering of Bcl-2 cleavage. To test this possibility, we treated H460 cells with different types of anticancer agents, such as paclitaxel and vinblastine, which are

antitubulin agents and which induce Bcl-2 phosphorylation. Other agents such as camptothecin, etoposide, and doxorubicin, which are Top I and Top II inhibitors and DNA intercalators, do not induce Bcl-2 phosphorylation. Under equal cytotoxic concentrations, all of the tested agents were able to produce the cleavage of Bcl-2 protein with the same molecular weight band for the cleavage product (M_r 23,000), and with a similar extent of cleavage (Fig. 3). These results indicate that the phosphorylation of Bcl-2 might not be a necessary event for the triggering Bcl-2 cleavage; that the phosphorylated Bcl-2 could alter its conformation and net charges may be another possibility for consideration. However, this type of modification of the Bcl-2 molecule may result in an easier attack by caspases and/or by other proteolytic factors. It is clear that the inhibition of the ubiquitin/proteasome pathway by PS-341 leads to Bcl-2 phosphorylation and to the generation of a unique fragment.

The effects of other types of protease inhibitors on Bcl-2 phosphorylation and cleavage suggested that only inhibitors of the chymotrypsin-related proteasome activity were capable of the induction of Bcl-2 phosphorylation and cleavage. Other protease inhibitors, such as calcium-dependent cysteine protease calpain inhibitors and apoptosis-related caspase inhibitors, were unable to induce Bcl-2 phosphorylation and cleavage. Treatment with the calpain inhibitors, ALLN and ALLM, caused a significant accumulation of Bcl-2 protein, which suggested that the degradation of Bcl-2 might be involved in the calpain protease-related pathways. Because the exposure to calpain inhibitors could increase the accumulation of Bcl-2 protein, the question of whether this could affect cellular susceptibility to anticancer agents was assessed. Preliminary results showed that treatment with 10 μM ALLN caused the reduction of cell sensitivity to doxorubicin and cisplatin in H460 cells (data not shown), which indicated that the inhibition of Bcl-2 degradation can partially elevate the threshold for cytotoxic agent-induced cell death. Thus, the findings with the calpain inhibitors suggest that the degradation of Bcl-2 protein may be generated through calpain-related neutral cysteine proteolytic pathways, although the data with the caspase inhibitors indicate that only proteasome inhibitors are capable of blocking the cell cycle at G₂-M phase, thereby inducing apoptosis, Bcl-2 phosphorylation, and cleavage.

There is evidence that Bcl-2 is an integration membrane protein, and resides in mitochondrial and endoplasmic membranes and nuclear envelope (44). It remains unknown whether Bcl-2 phosphorylation and cleavage by PS-341 could alter its subcellular distribution and whether the redistribution of Bcl-2 could be associated with the loss of its antiapoptotic function. Using immunocytochemistry, we did not detect any difference in Bcl-2 localization between treated and untreated cells. But, because the sensitivity of this technique is low, we then used a biochemical approach to determine Bcl-2 distribution. Intact and phosphorylated Bcl-2 was detected at both Triton X-100-soluble and -insoluble fractions. However, the cleaved fragment was distinctly at the Triton X-100-insoluble fraction, which suggested that the cleaved Bcl-2 protein was substantially attached at the membrane structures (Fig. 4). The functional significance of

the cleaved fragment at the membrane structure needs to be further investigated, but our data suggest that Bcl-2 phosphorylation did not markedly alter its subcellular distribution, and truncated Bcl-2 molecules are predominately localized at membrane structures.

A number of reports indicate that Bcl-2 protein is cleaved into a M_r 23,000 fragment by several stimuli such as virus infection, treatment with etoposide, and MG-132 (38–40). The treatment of H460 cells with PS-341 or with other kinds of chymotrypsin-related proteasome inhibitors resulted in a unique, additional M_r 25,000 product. This specific band occurred only in cells treated with proteasome inhibitors, not in cells treated with any kinds of anticancer agents, although a M_r 23,000 cleavage fragment was detected a result of their activity. The M_r 25,000 cleavage fragment was not mediated by any types of caspase pathways (Fig. 7), which indicated that the proteasome inhibitor induces a specific and unique cleavage fragment via the other proteolytic pathways. On the basis of the Bcl-2 structure, the COOH terminus is the membrane insertion domain, whereas, the NH_2 terminus at the BH4 domain is responsible for the interaction with apoptotic regulators. Because the cleavage of both M_r 23,000 and M_r 25,000 fragments may reside at the BH4 domain, it is worth considering whether the formation of M_r 25,000 fragments could be associated with the alteration in the apoptotic regulator function of the BH4 domain (38, 40, 45). All of the data with respect to the time course of events indicate that the cleavage of Bcl-2 is an early event, which precedes DNA fragmentation and the cleavage of PARP and β -catenin by at least 24 h. These results are consistent with the reports by Fadeel *et al.* (40).

Although the functional significance for M_r 25,000 cleaved fragment remains to be further investigated, the M_r 25,000 cleaved fragment was observed with *in vitro* and *in vivo* PS-341-treated PBMCs (Fig. 6). We feel that this could potentially be a useful pharmacodynamic marker for Phase II and I studies with PS-341.

According to our knowledge at present, there are two signaling pathways involved in the initiation of apoptosis cascades (46–48). One is via the same stimuli binding to apoptotic receptors, such as Fas and TNF receptor 1, and then through the activation of FADD/caspase-8 signaling to start the cascades of apoptosis (49). Another is the release of cytochrome c from mitochondria into cytoplasm by apoptotic stimuli. On release, cytochrome c binds to Apaf-1 and dATP, forming the apoptosome complexes, and activates effector procaspase-3 and caspase-7 (50). In our previous work, we found that PS-341 induces the cytochrome c release from mitochondria as early as 3–6 h after drug exposure and activation of caspase-8 and caspase-9 occurred at ~12 h, although the activation of casapse-3 and caspase-7 occurred at ~24 h. In this work, we found that Bcl-2 cleavage was at ~12 h and preceded the cleavages of PARP and β -catenin by ~24 h (Fig. 5). In addition, Bcl-2 cleavage was prevented by all types of caspase inhibitors except caspase-1 inhibitor; however, the cleavage of PARP was prevented only by the pan-caspase inhibitor and caspase-3 inhibitor (Fig. 7). This suggests that unlike PARP and β -catenin, Bcl-2 can be cleaved by various types of caspases,

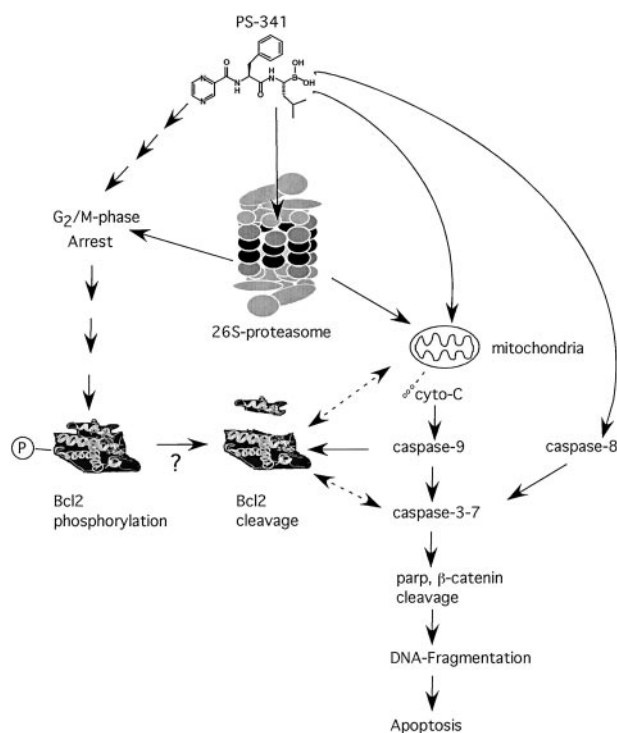


Fig. 8. Graphical schematic of the pleotropic activity of PS-341 showing parallel effects on the G_2 -M phase arrest that led to Bcl-2 phosphorylation and cleavage. Perturbations on mitochondria led to the release of cytochrome c and the subsequent cleavage of procaspases-9. The activated caspases-9 further cleaves procaspase-3 and -7 and subsequently leads to the cleavage of PARP and β -catenin, DNA-fragmentation, and apoptosis of the cell. The Bcl-2 can be cleaved by either caspases-9 at initiation stage or by caspases-3 or -7 at the execution stage. The Bcl-2 cleavage products can act on the mitochondria to induce cytochrome c release and further increase the activity of caspases-9, as well as of caspases-3 and -7 serving as a feed-back loop to amplify the PS-341-induced apoptotic cascades.

including caspase-8 and -9 at initiation phase. It is widely accepted that Bcl-2 protein can prevent the activation of caspases in the effective stage of apoptosis (8, 51). We have preliminary data showing that PS-341 treatment causes the cytochrome c release from mitochondria and could then induce the activation of initiators of the caspase pathway. Fig. 8 shows a graphic summary of observed points of activity of PS-341 based on our new observations with H460 cells. One working hypothesis is that, the activated caspases (-3, -7, and -9) through PS-341 effects on mitochondria, could cleave Bcl-2 and convert its antiapoptotic form to the truncated Bcl-2 molecule with a pro-apoptotic function. The cleaved Bcl-2 could induce cytochrome c release from mitochondria and the subsequent activation of caspases-3 and -7. The released cytochrome c and activated caspase-3 and -7 would further enhance the cleavage of Bcl-2 and other target proteins. Such a feedback loop could lead to an amplification of PS-341-induced apoptotic cascades. Our data indicate that Bcl-2 protein can serve as a substrate for the initiator and effector caspases. However, the generation of the M_r 25,000 fragment was not mediated by a caspase-dependent pathway.

It is conceivable that PS-341 treatment causes the cytochrome *c* release from mitochondria and, subsequently, the activation of initiators of caspase. One working hypothesis could be that the activated caspases could cleave Bcl-2 and convert its antiapoptotic form to the truncated Bcl-2 molecule with a proapoptotic function. Furthermore, the cleaved Bcl-2 could further induce cytochrome *c* release from mitochondria, and further activation of caspases-3. The released cytochrome *c* and activated caspase-3 would further enhance the cleavage of Bcl-2 and other target proteins. Such a feed-back loop could lead to an amplification of PS-341-induced apoptotic cascades. Overall, our data indicate that Bcl-2 protein can serve as a substrate for the initiator and effector caspases and that generation of the *M_r* 25,000 fragment was not mediated by a caspase-dependent pathway.

References

- Wyllie, A. H., Kerr, J. F. R., and Currie, A. R. Cell death: the significance of apoptosis. *Int. Rev. Cytol.*, **68**: 251–305, 1980.
- Vaux, D. L., and Korsmeyer, S. J. Cell death in development. *Cell*, **96**: 245–254, 1999.
- White, E. Life, death, and the pursuit of apoptosis. *Genes Dev.*, **10**: 1–15, 1996.
- Scaffidi, C., Kirchhoff, S., Kramer, P. H., and Peter, M. E. Apoptosis signaling in lymphocytes. *Curr. Opin. Immunol.*, **11**: 277–285, 1999.
- Gejewski, T. F., and Thompson, C. B. Apoptosis meets signal transduction of a BAD influence. *Cell*, **67**: 589–592, 1996.
- Brenner, C., and Kroemer, G. Apoptosis, mitochondria—the death signal integrators. *Science (Wash. DC)*, **289**: 1150–1151, 2000.
- Bakhshi, A., Jensen, J. P., Goldman, P., Wright, J. J., McBride, O. W., Epstein, A. L., Korsmeyer, S. J. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around *J_H* on chromosome 14 and near a transcriptional unit on 18. *Cell*, **41**: 889–906, 1985.
- Kroemer, G. The proto-oncogene *Bcl-2* and its role in regulating apoptosis. *Nat. Med.*, **3**: 641–620, 1997.
- Reed, J. C., Miyashita, T., Takayama, S., Wang, H-G., Sato, T., Krajewski, S., Aime-Sempe, C., Bodrug, S., Kitada, S., and Hanada, M. Bcl-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. *J. Cell. Biochem.*, **60**: 23–32, 1996.
- Cryns, V., and Yuan, J. Proteases to die for. *Genes Dev.*, **12**: 1551–1570, 1998.
- Gross, A., McDonnell, J. M., and Korsmeyer, S. J. Bcl-2 family members and the mitochondria in apoptosis. *Genes Dev.*, **13**: 1899–1911, 1999.
- Mikhailov, V., Mikhailova, M., Pulkrabek, D. J., Dong Z., Venkatachalam, M. A., and Saikumar, P. Bcl-2 prevents Bax oligomerization in the mitochondrial outer membrane. *J. Biol. Chem.*, **271**: 18361–18374, 2001.
- Yang, E., and Korsmeyer, S. J. Molecular thanatopsis: a discourse on the BCL2 family and cell death. *Blood*, **88**: 386–401, 1996.
- Haldar, S., Jena, N., and Croce, C. M. Inactivation of Bcl-2 by phosphorylation. *Proc. Natl. Acad. Sci. USA*, **92**: 4507–4511, 1995.
- Blagosklonny, M. V., Giannakou, P., El-Deiry, W. S., Kingston, D. J. I., Higgs, P. I., Neckers, L., and Fojo, T. Raf-1/bcl-2 phosphorylation; a step from microtubule damage to cell death. *Cancer Res.*, **57**: 130–135, 1997.
- Fang, G., Chang, B. S., Kim, C. N., Perkins, C., Thompson, C. B., and Bhalla, K. N. “Loop” domain is necessary for Taxol-induced mobility shift and phosphorylation of Bcl-2 as well as for inhibiting Taxol-induced cytosolic accumulation of cytochrome *c* and apoptosis. *Cancer Res.*, **58**: 3202–3208, 1998.
- Fan, M., Goodwin, M., Vu, T., Brantley-Finley, C., Gaarde, W. A., and Chambers, T. C. Vinblastine-induced phosphorylation of Bcl-2 and Bcl-XL are mediated by JNK and occur in parallel with inactivation of Raf-1/MEK/ERK cascade. *J. Biol. Chem.*, **275**: 29980–29985, 2000.
- May, W. S., Tyler, P. G., Ito, T., Armstrong, D. K., Qatsha, K. A., and Davidson, N. E. Interleukin-3 and bryostatin-1 mediated hyperphosphorylation of BCL2 α in association with suppression of apoptosis. *J. Biol. Chem.*, **269**: 26865–26870, 1994.
- Poommipanit, P., Chen, B., and Oltvai, Z. N. Interleukin-3 induces the phosphorylation of a distinct fraction of bcl-2. *J. Biol. Chem.*, **274**: 1033–1039, 1999.
- Srivastava, R. K., Mi, Q. S., Hardwick, J. M., and Longo, D. Deletion of the loop region of Bcl-2 completely blocks paclitaxel-induced apoptosis. *Proc. Natl. Acad. Sci. USA*, **96**: 3775–3780, 1999.
- Ito, T., Deng, X., Carr, B., and May, W. S. Bcl-2 phosphorylation required for anti-apoptosis function. *J. Biol. Chem.*, **272**: 11671–11673, 1997.
- Ling, Y. H., Tomos, C., and Perez-Soler, R. Phosphorylation of Bcl-2 is a marker of M phase events and not a determinant of apoptosis. *J. Biol. Chem.*, **273**: 18984–18991, 1998.
- Cheng, EH-Y, Kirsch, D. G., Clem, R. J., Ravi, R., Kastan, M. B., Bedi, A., Ueno, K., and Hardwick, J. M. Conversion of bcl-2 to a bax death effector by caspases. *Science (Wash. DC)*, **278**: 1966–1968, 1997.
- Hershko, A., and Ciechanover, A. The ubiquitin system. *Annu. Rev. Biochem.*, **67**: 425–480, 1998.
- Juntsch, S., and Schlenker, S. Selective protein degradation: a journey's end within the proteasome. *Cell*, **82**: 881–884, 1995.
- Glotzer, M., Murray, A. W., and Kirschner, M. W. Cyclin is degraded by the ubiquitin pathway. *Nature (Lond.)*, **349**: 132–138, 1991.
- Ciechanover, A., DiGiuseppe, A. J., Bercovich, B., Orian, A., Richter, J. D., Schwartz, A. L., and Brodeur, G. M. Degradation of nuclear oncoproteins by the ubiquitin system *in vitro*. *Proc. Natl. Acad. Sci. USA*, **88**: 139–143, 1991.
- Yang, Y., Fang, S., Jensen, J. P., Weissman, A. M., and Ashwell, J. D. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science (Wash. DC)*, **288**: 874–877, 2000.
- Grimm, L. M., Gildberg, A. L., Porier, G. G., Schwartz, L. M., and Osborne, B. A. Proteasomes play an essential role in thymocyte apoptosis. *EMBO J.*, **15**: 3835–3844, 1996.
- Lopes, U. G., Erhardt, P., Yao, R., and Cooper, G. M. p53-dependent induction of apoptosis by proteasome inhibitors. *J. Biol. Chem.*, **272**: 12893–12896, 1997.
- Shinohara, K., Tomioka, M., Nakano, H., Tone, S., Ito, H., and Kawashima, S. Apoptosis induction resulting from proteasome inhibition. *Biochem. J.*, **317**: 385–388, 1996.
- Drexler, H. C., Risau, W., and Konecny, M. A. Inhibition of proteasome function induces programmed cell death in proliferating endothelial cells. *FASEB J.*, **14**: 65–77, 2000.
- Adams, J., Behnke, M., Chen, S., Cruickshank, A. A., Dick, L. R., Grenier, L., Klunder, J. M., Ma, Y-T., Plamondon, L., and Stein, R. L. Potent and selective inhibitors of the proteasome: dipeptidyl boronic acids. *Bioorg. Med. Chem. Lett.*, **8**: 333–338, 1998.
- Adams, J., Palombella, V. J., Sausville, E. A., Jonson, J., Destree, A., Lazarus, D. D., Maas, J., Pien, C. S., Prakash, S., and Elliott, P. J. Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer Res.*, **59**: 2615–2622, 1999.
- Ling, Y. H., Priebe, W., and Perez-Soler, R. Apoptosis induced by anthracycline antibiotics in P388 parent and multidrug-resistant cells. *Cancer Res.*, **53**: 1845–1852, 1993.
- Lampugnani, M. G., Corada, M., Caveda, L., Breviario, F., Ayalon, O., Geiger, B., and Dejana, E. The molecular organization of endothelial cells to cell junctions: differential association of plakoglobin, β -catenin, and α -catenin with vascular endothelial cadherin (VE-cadherin). *J. Cell Biol.*, **129**: 203–217, 1995.
- Perez-Soler, R., Ling, Y. H., Elliott, P. J., Adams, J., and Liebes, L. Effect of the proteasome inhibitor PS-341 on cell cycle progression and bcl-2: a potentially unique mechanism of action. *Clin. Can. Res.*, **6**: 4549s, 2000.
- Grandgirard, D., Studer, E., Monney, L., Belser, T., Fellay, I., Borner, C., and Michel, M. R. Alphaviruses induce apoptosis in Bcl-2 overexpress-

- ing cells: evidence for a caspase-mediated, proteolytic inactivation of Bcl-2. *EMBO J.*, 17: 1268–1278, 1998.
39. Zhang, X.-M., Lin, H., Chen, C., and Chen, B. D.-M. Inhibition of ubiquitin-proteasome pathway activates caspase-3-like protease and induces Bcl-2 cleavage in human M-07e leukaemic cells. *Biochem. J.*, 340: 127–133, 1999.
40. Fadeel, B., Hassan, Z., Hellstrom-Lindberg, E., Henter, J.-I., Orrenius, S., and Zhivotovsky, B. Cleavage of Bcl-2 is an early event in chemotherapy-induced apoptosis of human myeloid leukemia cells. *Leukemia (Baltimore)*, 13: 719–728, 1999.
41. Yamamoto, K., Ichijo, H., and Korsmeyer, S. J. Bcl-2 phosphorylation and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G₂-M. *Mol. Cell. Biol.*, 19: 8469–8478, 1999.
42. Pathan, N., Aime-Sempe, C., Kitada, S., Halder, S., and Reed, J. C. Microtubule-targeting during induce Bcl-2 phosphorylation and association with Pin 1. *Neoplasia*, 37: 10–19, 2001.
43. Blagosklonny, M. V. Unwinding the loop of Bcl-2 phosphorylation. *Leukemia (Baltimore)*, 15: 869–874, 2001.
44. de Jong, D., Prins, F. A., Mason, D. Y., Reed, J. C., van Ommen, G. B., and Kluijn, P. M. Subcellular localization of the bcl-2 protein in malignant and normal lymphoid cells. *Cancer Res.*, 54: 256–260, 1994.
45. Kelekar, A., and Thompson, C. B. Bcl-2-family protein: the role of the BH3 domain in apoptosis. *Trends Cell Biol.*, 8: 324–330, 1998.
46. Li, H., and Yuang, J. deciphering the pathways of life and death. *Curr. Opin. Cell Biol.*, 11: 261–266, 1999.
47. De Maria, R., Testa, U., Luchetti, L., Zeuner, A., Stassi, G., Pelosi, E., Riccioni, R., Felli, N., Samoggia, P., and Peschle, C. Apoptotic role of Fas/Fas ligand system in the regulation of erythropoiesis. *Blood*, 93: 796–803, 1999.
48. Waterhouse, N. J., and Green, D. R. Mitochondria and apoptosis: HQ or high-security prison? *J. Clin. Immunol.*, 19: 378–387, 1999.
49. Luschen, S., Ussat, S., Scherer, G., Kabelitz, D., and Adam-Klages, S. Sensitization to death receptor cytotoxicity by inhibition of Fas-associated death domain protein (FADD)/caspase signaling. *J. Biol. Chem.*, 275: 24670–24678, 2000.
50. Li, P., Nijhawan, D., Budihardjo, I., Srinivasila, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. Cytochrome c and dATP-dependent formation of apaf-1/caspase-9 complex initiates apoptotic protease cascade. *Cell*, 91: 474–489, 1997.
51. Kluck, R. M., Bossy-Wetzell, E., Green, D. R., and Newmeyer, D. D. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science (Wash. DC)*, 275: 1132–1136, 1997.