The proteasome inhibitor PS-341 inhibits growth and induces apoptosis in Bcr/Abl-positive cell lines sensitive and resistant to imatinib mesylate

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Background and Objectives. Imatinib mesylate (IM) is the choice treatment for Bcr/Abl-positive malignancies. Emergence of resistance to IM warrants the exploration of novel well-tolerated anticancer agents. We intended to evaluate the effect of PS-341 on proliferation, survival, and cellular events in Bcr/Abl-positive cells sensitive and resistant to IM, and to investigate the effect of PS-341 and IM in conjunction.

Design and Methods. Bcr/Abl-positive cell lines sensitive (p210Bcr/Abl KBM5, p210Bcr/Abl KBM7, and p190Bcr/Abl Z-119) or resistant (KBM5-R) to IM were treated with PS-341 alone or in combination with IM. The effect on cell growth was determined using the MTT assay. Cell-cycle analysis was performed by propidium iodide staining. Apoptosis was evaluated by measurement of sub-G1 DNA content, annexin V binding, and caspase 3 activity assays. Levels of apoptotic proteins, P-IkBα, Bcr/Abl, and phosphorylated Bcr/Abl were determined by western blotting. NF-κB activity was evaluated by electromobility gel shift assays.

Results. PS-341 exerted growth inhibition effects in IM-sensitive and -resistant cells. This phenomenon correlated with accumulation of cells in the G2/M phase of cell cycle; transient downregulation of NF-κB DNA binding activity; downregulation of Bcl-xL; activation of caspase 3; induction of apoptosis; inhibition of expression and phosphorylation of Bcr/Abl. Sequential combination of PS-341 followed by IM demonstrated a synergistic pro-apoptotic effect in IM-sensitive cells; concomitant exposure was antagonistic.

Interpretation and Conclusions. PS-341 suppresses growth and induces apoptosis in Bcr/Abl-positive cells sensitive and resistant to IM. The use of PS-341 should be explored in patients with chronic myelogenous leukemia resistant to IM. Trials of combinations of PS-341 and IM require cautious design.

Key words: CML, PS-341, STI571-resistant cell lines.

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cell line with proteasome inhibitors has been reported to induce significant reduction of Bcr/Abl protein expression and its autophosphorylation, with subsequent induction of apoptosis. Thus, these drugs may have potential to overcome IM resistance associated with overexpression of Bcr/Abl as well as with mutations within the critical ATP/IM binding pocket, recently described in CML cell lines and in patients resistant to IM.

PS-341, a boronic acid dipeptide derivative with a high degree of selectivity for the proteasome, showed cytotoxicity against a broad range of human tumor cell lines in vitro. In human multiple myeloma cells, PS-341 inhibited growth, induced apoptosis, and overcame drug resistance to doxorubicin, mitoxantrone, and melphalan. Incubation of human colorectal cancer cells with PS-341 enhanced the cells' chemosensitivity to CPT-11. In human xenografts, a marked in vivo activity of PS-341 was seen against human prostate cancer, breast cancer, and squamous cell carcinoma. In a preclinical tumor model of breast cancer, PS-341 increased the rate of tumor cell killing by radiation therapy, cyclophosphamide, and cisplatin. PS-341 also showed additive growth delays with 5-fluorouracil, cisplatin, paclitaxel, and doxorubicin against Lewis lung carcinoma. In this study, we examined the effect of PS-341 on proliferation, survival, and cellular events in Bcr/Abl-positive leukemia cell lines sensitive to IM, as well as in a resistant subline established by repeated exposure to IM in vitro. In this model, resistance to IM was associated with overexpression of Bcr/Abl protein and mutation in the ATP binding domain of ABL, resulting in increased kinase activity. We also investigated the potential advantage of combining PS-341 with IM.

Design and Methods

Drugs

The proteasome inhibitor PS-341 [pyrazyl-CONH(CHPhe)CONH(Chisobutyl)BOH], provided by Millenium Predictive Medicine Inc. (Cambridge, MA, USA), was dissolved in dimethylsulfoxide and stored as a 50 mM stock concentration at –20°C until used. STI571 (imatinib mesylate) was provided by Ciba-Geigy (now Novartis, Basel, Switzerland), and stored as a 10 mM stock concentration at –20°C. Fresh working solutions in 1× phosphate-buffered saline (PBS) were prepared before each experiment.

Myeloid leukemia cell lines

Except for HL60, which was obtained from the American Type Culture Collection (Rickville, MD, USA), the cell lines used in this study were established in our laboratory. The KBM-5 and KBM-7 cell lines were derived from patients with CML in myeloid blastic phase. The KBM-5 cell line expresses p210Bcr/Abl and lacks normal c-ABL. The KBM-7, a near-haploid cell line, also contains the Ph chromosome and expresses p210Bcr/Abl. IM-resistant cells, designated KBM-5-STI571 and here referred to as KBM-5R, were established by growing KBM-5 parental cells in the presence of gradually increasing concentrations of imatinib mesylate (up to 1 μM). Compared with KBM-5 parental cells, KBM-5R cells are characterized by a marginal increase in the BCR/ABL copy number, a modest increase in p210 expression, and the presence of a single change C-T at ABL nucleotide 944 (T315I) in a proportion of BCR/ABL amplicons.

Lymphoblastic leukemia cell line

The Z-119 cell line was kindly provided by Dr. Zeev Estrov (Department of Bioimmunotherapy). This cell line, which was derived from a Ph-chromosome positive patient with ALL, retains typical B-cell characteristics and phenotype and expresses p190Bcr/Abl.

Cell cultures

All cell lines were cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal calf serum (Invitrogen Corp., Carlsbad, CA, USA).

Growth inhibition assays

The inhibitory effect of PS-341 on the growth of cell lines was assessed using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] colorimetric dye reduction method (Sigma Chemical Co., St. Louis, MO, USA). Cells were plated in triplicate in 96-well plates at a concentration of 0.4×10⁶ cells/mL of medium. PS-341 was added at various concentrations, and growth inhibition was measured after 48 to 120 h of exposure. Inhibition of proliferation was evaluated as a fraction of colony growth in drug-free media. In simultaneous combination experiments, the two drugs were added concurrently. In sequential combination experiments, cells were exposed to 11 nM PS-341 for 12 h, washed twice with PBS to remove the drug, plated in triplicate in 96-well plates at a concentration of 0.4×10⁶ cells/mL of medium and then exposed to imatinib mesylate at various concentrations. Growth inhibition was measured after 72 h.

Growth inhibition as a function of drug exposure time

The kinetics of growth inhibition as a function of drug exposure time and the reversibility of the PS-341 effect on cell growth were studied in the KBM-5 cell line. Cells were grown in the presence of PS-341 at various concentrations or in drug-free medium for 12, 24, 36, or 48 h and then washed twice with PBS and cultured in PS-341-free medium for 48 h. Every 12 h, viable cells were counted in a counting chamber after being stained with trypan blue.
**Cell cycle and apoptosis analysis**

Cells were cultured in drug-free medium or in the presence of PS-341 at various concentrations for 12, 24, or 48 h. Subsequently, the cells were fixed in ice-cold ethanol (70% v/v) and stained with a solution of propidium iodide (PI) (25 µg/mL PI; 180 µM RNase; 0.1% Triton X-100; and 30 mg/mL polyethylene glycol in 4 mM citrate buffer, pH 7.8; Sigma Chemical Co.). The DNA content was determined using a FACScan flow cytometer (Becton Dickinson, San José, CA, USA). Cell-cycle distribution was analyzed using ModFit LT software (Verity Software House, Topsham, ME, USA). Cells with a hypodiploid DNA content (<2 n and >0.2 n) were counted as apoptotic. For annexin V binding studies, cells were washed twice with cold 1X PBS buffer, incubated with a 1:100 solution of FITC-conjugated annexin V (Trevigen Inc., Gaithersburg, MD, USA) for 15 min at room temperature, and 400 µL 1X binding buffer (10 mM HEPES, pH 7.4; 150 mM NaCl; 1.8 mM CaCl2; 5 mM KCl; 1 mM DTT; and 0.1% CHAPS; Trevigen Inc.) were then added. Cells were analyzed by flow cytometry; simultaneously, membrane integrity was assessed by PI exclusion. In subsequent combination experiments, cells were pretreated with 11 nM PS-341 for 12 h, washed twice with PBS to remove the drug, and then exposed to IM at various concentrations for 72 h before annexin V binding studies. In simultaneous combination experiments, the two drugs were added concurrently and analysis of apoptosis was performed at 48 h.

**Caspase 3 assay**

Caspase 3 activity was assayed with an Apoalert Caspase 3 Colorimetric Assay kit (Calbiochem, San Diego, CA, USA). In brief, control and treated cells were washed with cold PBS and lysed in lysis buffer containing 50 mM HEPES, pH 7.4; 100 mM NaCl; 0.1% CHAPS; 1 mM dithiothreitol (DTT); and 0.1 mM EDTA for 10 minutes and then centrifuged at 10,000 xg for 20 minutes before collecting the supernatants. Fifty micrograms of each lysate were added to the assay buffer (50 mM HEPES, pH 7.4; 100 mM NaCl; 0.1% CHAPS; 1 mM DTT; 0.1 mM EDTA; and 10% glycerol) to make a total volume of 90 µL which was then incubated at 37°C for 10 minutes. Ten microliters of colorimetric substrate for caspase 3 (final concentration, 200 µM) were added to the mixtures. A405 was recorded for each sample after incubation at 37°C for 2 h.

**Electrophoretic mobility shift assays (EMSA)**

Cells were washed with cold PBS, resuspended in buffer A (10 mM Hepes, pH 7.9; 10 mM KCl; 0.1 mM EDTA, pH 8; 1 mM DTT; 1 mM phenylmethylsulfonyl fluoride (PMSF); 2 µg/mL leupeptin; 2 µg/mL aprotinin; and 0.5 µg/mL benzamidine) for 15 minutes and lysed with 10% NP-40. Nuclei were separated by centrifugation at 4,000 xg for 3 minutes in a micro-centrifuge. Cytoplasmic proteins were collected and stored at −80°C for future usage. nuclei were resuspended in buffer B (20 mM Hepes, pH 7.9; 400 mM NaCl; 1 mM EDTA, pH 8; 1 mM DTT; 1 mM PMSF; 2 µg/mL leupeptin; 2 µg/mL aprotinin, and 0.5 mg/mL benzamidine), vortexed, and incubated on ice for 30 minutes. The lysed nuclei were centrifuged for 20 minutes at 4°C at 14,000 xg in a micro-centrifuge, and the nuclear extracts were assayed for protein using the Biorad assay method and stored at −80°C. Gel shift assays were performed according to the gel shift assay protocol from Promega Corp. (San Luis Obispo, CA, USA). A 22-mer double-stranded–NF-κB consensus oligonucleotide, 5’AGT TGA GGG GAC TTT CCC AGG C-3’, was labeled with γ32P. For each reaction, 10 µg of nuclear extract were incubated with 2 µL of 5X binding buffer, 2 µL of poly(dI:dC) (1 µg/µL), 2 µL of 10% NP-40, and 2 µL of the 32P-labeled oligo at 37°C for 15 minutes. The samples were then subjected to 6% non-denaturing acrylamide gel electrophoresis, and the gel was dried and exposed to a phosphoimagining plate for at least 12 h and then visualized with a phosphorimagger. For EMSA supershift analysis, 1 µL of the NF-κB supershift antibodies for p50 and p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added to the nuclear extract 15 minutes before the labeled probe was added. For specificity, 1 µL of unlabelled consensus oligonucleotide was used to compete out the labeled probe. For supershift analysis, the samples were subjected to a 4.5% non-denaturing acrylamide gel.

**Western blot analysis**

For detection of Bcr–Abl, phosphorylated proteins and p27, cells were washed twice with cold PBS containing protease inhibitors and phosphatase inhibitors (protease inhibitor cocktail [Complete, Mini Roche Molecular Biochemical, Indianapolis, IN, USA], 1 mM PMSF, 1 mM ortovanadate, 10 mM sodium fluoride, 10 µg/mL leupeptin and 10 µg/mL apro tinin), and 1X107 cells were lysed in 1 mL of lysis buffer (0.125 M Tris–HCl, pH 6.8; 1% SDS, 0.01% bromophenol blue; 5% glycerol; 2% 2-mercaptoethanol; and protease and phosphatase inhibitors). Cell lysates corresponding to 5X106 cells were boiled for 10 min, resolved on SDS–PAGE gels, transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA), and immunoblotted with anti-c–Abl, anti-p27 (Pharmingen, San Diego, CA, USA), or anti-phosphotyro sine (4G10 monoclonal; Upstate Biotechnology, Lake Placid, NY, USA). For detection of P–IκBα, Bax, Bcl–2, and Bcl–xL, cytoplasmic proteins were collected as described above and quantified using the Biorad assay method. Fifty micrograms of cytoplasmic
lysates were subjected to SDS-PAGE gels, transferred onto PVDF membranes and immunoblotted with anti-P-IkBα (Cell Signaling Technology, Beverly, MA, USA), anti-Bax, anti-Bcl-xL (Santa Cruz Biotechnology), or anti-Bcl-2 (Upstate Biotechnology). After overnight incubation at 4°C with the primary antibody, the membranes were washed and incubated with secondary anti-mouse (Bio-Rad, Hercules, CA, USA) or anti-rabbit (Upstate Biotechnology) horseradish peroxidase-conjugated antibodies at room temperature for 1 h. The membranes were washed, and bound antibodies were detected with enhanced luminal and oxidizing reagent by chemiluminescence as specified by the manufacturer (Amersham, Arlington Heights, IL, USA). After stripping the membranes in the stripping buffer (0.5 mM Tris-HCl, pH 6.7; 2% SDS; and 100 mM 2-mercaptoethanol) for 30 min at 56°C, they were re-probed with anti β-actin antibodies (Sigma, Chemical Co.) to assess the comparability of the protein loading.

**Statistical evaluation of drug combinations**

Multiple linear regression was used to explore the relationship between the explanatory variables and the response. The mean response was assumed to be a linear function of the dose of imatinib mesylate, the dose of PS341, and the interaction of the two agents. The full model was tested with the analysis of variance F test. Tests to determine corresponding significance were performed for each of the regression coefficients. The studies were based on the rationale that if the effect of imatinib mesylate depended on the use of PS341, there would be an interaction between the two drugs: synergy would result in a significant interaction effect (p < 0.05) that would have the same sign (+ or -) as that of the main effects of the two drugs, whereas a significant interaction effect whose sign was opposite to that of the two drugs would suggest an antagonistic relationship. If the interaction effect was not significant (p ≥ 0.05), additive treatment would be appropriate to characterize the relationship between the two drugs used in combination. The effects were graphically displayed with interaction plots. All computations were carried out using SAS release 8.01 (SAS Institute Inc, Cary, NC, USA).

**Results**

**Effects of PS-341 on growth of human leukemic cell lines**

Using the MTT assay, we determined the effect of PS-341 on the growth of human leukemic cell lines (KBM-5, KBM-7, HL60, and Z-119) cultured for 48, 72, or 120 h. After 48 h, 50% growth inhibition (IC50) was noted in HL60, KBM-5, and KBM-7 cells at concentrations ranging from 10 to 15 nM (Figure 1A). In the Z-119 cell line (p190Bcr/Abl), which was evaluated after 120 h in culture because of its slower rate of proliferation, the IC50 of PS-341 was 8 nM (Figure 1B). The possibility of a cross-resistance between PS-341 and IM was evaluated by comparing the effect of PS-341 on the growth of KBM-5 and KBM-5R. As shown in Figure 1C, the dose-response curves for KBM-5R and KBM-5 cells were essentially identical, indicating the lack of cross-resistance between IM
and PS-341.

To determine the reversibility of drug action, we studied the effect of PS-341 in KBM-5 cells as a function of the duration of the exposure followed by standard time culture after wash out of the drug. Cells exposed to a PS-341 concentration of 8 nM for up to 48 h were able to regrow in the drug-free medium (data not shown). Cells exposed to 11 nM of PS-341 for 12 h were able to regrow in the drug-free medium at the same rate as that seen in the untreated control, whereas cells exposed for longer periods showed only partial growth recovery in the drug-free medium (Figure 2). Cells exposed to 20 nM PS-341 showed complete abrogation of growth after only 12 h of drug exposure (data not shown).

**Effects of PS-341 on cell cycle and apoptosis**

For further analysis of the mechanism of growth inhibition induced by PS-341, we examined the cell cycle and the apoptosis profile of KBM-5 cells cultured either in drug-free medium or in the presence of PS-341 at different concentrations (1, 5, 10, 15, or 20 nM) for 12, 24, or 48 h. PS-341 induced a dose-dependent accumulation of cells in the G2/M phase of the cell cycle and an increase in the rate of apoptosis as evaluated by annexin V binding and measurement of sub-G0 DNA content (Figure 3A and B).

The correlation between the proportional accumulation of the PS-341-treated cells in the G2/M phase of the cell cycle and the percentage of sub-G0/G1 cells is shown by the results of a representative experiment in Figure 3C. It documents that accumulation of cells in the G2/M phase precedes the increase in the rate of apoptosis by 12–24 hours.

**Constitutive activation of NF-κB in Bcr/Abl-positive cell lines**

Using EMSA we confirmed the constitutive activation of NF-κB in IM-sensitive (KBM-5, KBM-7, and Z-119) and IM-resistant (KBM-5R) cell lines (Figure 4A). A supershift assay performed by incu-
bating the nuclear extracts of KBM-5 and KBM-5R cell lines with anti-p50 and anti-p65 antibodies showed the presence of these two subunits of the NF-κB proteins bound in the shifted complex (data not shown). To explore the hypothesis that Bcr/Abl induces NF-κB activity, we studied the effects of 1 µM IM on NF-κB DNA binding activity in KBM-5 and KBM-5R cells after 12, 24, and 48 h of exposure. As shown in Figure 4B, after 48 h of exposure, IM induced a clear weakening of NF-κB DNA binding activity in the parental but not in the IM-resistant cells.

Effects of PS-341 on P-IκBα degradation and NFκB DNA binding activity

We then investigated whether PS-341 inhibited the proteasome-mediated degradation of the phosphorylated form of IκBα, thereby impairing the translocation of NF-κB into the nucleus. KBM-5 and KBM-5R cells were exposed to 25 nM PS-341 for 3, 12, and 24 h. Cytoplasmic extracts were obtained and immunoblotted with anti-P-IκBα antibody, whereas nuclear extracts were used to examine the effect of PS-341 on NF-κB DNA binding by EMSA. As shown in Figure 4C and D, PS-341 induced accumu-
loration of the phosphorylated form of IκBα at 12 h in KBM-5 cells, which correlated with a reduction of NF-κB DNA binding activity in the nucleus at the same time point. In KBM-5R, the same experiment suggested a marginal decrease of the NF-κB DNA binding activity after 3 h of exposure to PS-341, which was not associated with accumulation of P-IκBα in the cytoplasm at the same time point. The effects were transient in both cell lines; after longer times of exposure, P-IκBα was degraded and the NF-κB DNA binding activity was restored.

**Effect of PS-341 on Bcr/Abl protein expression and phosphorylation**

As documented by Western blot analysis, exposure of KBM-5 and KBM-5R cells to PS-341 25 nM reduced both the levels of expression and phosphorylation of Bcr/Abl. While the effect was detectable already after 12 h of exposure, an almost complete abrogation was seen after 24 h (Figure 5). The effect was dose-dependent, as lower concentrations of the drug (11 nM and 20 nM) induced weaker and transient downregulation of Bcr/Abl expression and phosphorylation (*data not shown*).

**Effects of PS-341 on the expression of apoptotic proteins**

In both KBM-5 and KBM-5 R cell lines, we analyzed effects of PS-341 on the expression of several apoptosis-related proteins. Induction of caspase 3 activity was first detected after 6 h of treatment with 25 nM of PS-341 and the activity continued to increase during the 24 h observation period (Figure 6). The drug induced no changes in Bcl-2 or Bax expression, whereas a downregulation of Bcl-XL was evident after 24 h of drug exposure (Figure 5). Evaluation of p27 protein levels in both cell lines failed to reveal any significant changes associated with PS-341 treatment (*data not shown*).
Effects of the combination of PS-341 with imatinib mesylate

To determine whether PS-341 was able to enhance the effects induced by IM, we evaluated the combination of the two drugs in KBM-5 and KBM-5R cells by annexin V binding and the MTT assay. Antagonistic effects were observed when PS-341 and IM were added simultaneously in KBM-5, while in KBM-5R the combination of the two drugs produced the same results as PS-341 alone (data not shown). We then explored the possibility of sensitizing BCR/ABL-positive cell lines to IM by pre-exposing them for a short period to PS-341. In particular, we sought to use PS-341 at concentrations low enough not to cause irreversible cytotoxic effects. On the basis of results of growth inhibition as a function of drug exposure time, we elected to pre-expose the cells for 12 h to 11 nM PS-341, before treatment with various concentrations of IM. The combined effects of the two drugs on cell growth and on induction of apoptosis were evaluated in KBM-5 cells, we observed additive effects of growth inhibition, as evaluated by the MTT assay, for doses of IM ≤ 0.5 mM (p = 0.05) (Figure 7A). For doses of IM > 0.5 mM, there was an apparent antagonism due to the model system used and to the MTT assay: 1) in order to show additive effects, the expected fraction of surviving cells with the combination of high doses of IM with PS-341 would be less than 0%; and 2) the MTT assay is unable to detect levels of cell viability below 10% reliably. However, sequential exposure of cells to the two drugs resulted in evident synergistic pro-apoptotic effects, as evaluated by annexin V binding (p < 0.01) (Figure 7B). Even though PS-341 was equally active in KBM-5R and KBM-5 cells (Figure 1), at subtoxic doses it was unable to sensitize the resistant cells to IM, in terms of growth inhibition and apoptosis (Figure 7 C and D).

Discussion

Inhibition of the degradation of multiubiquinated target proteins involved in the regulation of cell cycle progression and apoptotic cell death by proteasome inhibitors has recently been proposed as a potential novel and effective approach to cancer therapy. Among several proteasome inhibitors, PS-341 has been shown to have significant cytotoxicity against a broad range of human tumor cells; PS-341 was also shown to be highly selective, directly affecting its biochemical target.

We demonstrated that PS-341 exerts potent time- and dose-dependent growth inhibition effects on both Ph- (HL60) and Ph+ (KBM-5, KBM-7 and Z119) cell lines with efficacy at similar concentrations. We have recently documented that IM-resistant KBM-5R cells overexpress the Bcr/Abl protein and carry a mutation within the coding sequence for the ATP binding pocket of the BCR/ABL fusion gene. We demonstrated that PS-341 was equally effective in suppression of cell growth and induction of apoptosis in both KBM-5 and in KBM-5R sublines at similar concentrations, suggesting lack of cross-resistance between IM and PS-341. These observations are potentially relevant to the use of PS-341 in clinical trials in patients with CML who have become resistant to IM.
In agreement with previous reports, we observed that the growth-inhibitory effects of PS-341 were due to an accumulation of the cells in the G2/M phase of the cell cycle, followed by a progressive increase in the rate of apoptotic cell death. It has been recently reported that treatment of CML cell lines with proteasome inhibitors induces significant reduction of Bcr/Abl protein expression and kinase activity, with subsequent induction of apoptosis. In our experiments, PS-341-induced apoptosis was associated with a decrease in Bcr/Abl expression and phosphorylation, downregulation of Bcl-XL, and activation of caspase 3, without demonstrable changes in Bcl-2 or Bax expression. Since resistance to IM is intimately associated with the level of Bcr/Abl expression and tyrosine kinase activity, PS-341-induced suppression of this activity may be sufficient to induce apoptotic death of IM-resistant cells. Bcr/Abl protection from apoptotic cell death is partially accomplished through the NF-κB pathway.

The proteasome inhibitor, PS-341, has been shown to block proteasome-mediated degradation of IκB, resulting in inhibition of NF-κB activity. We, therefore, anticipated that the effect of PS-341 on Bcr/Abl-positive cell lines may involve the IκB/NF-κB pathway.

With the finding that the PS-341-induced drop of NF-κB DNA binding activity was only transient and without a clear correlation of a decline in the NF-κB DNA binding activity with induction of apoptosis, we conclude that IκB/NF-κB is not the main factor responsible for the effects observed. Furthermore, the levels of P- IκB did not correlate with alterations in NF-κB DNA binding activity. There are two possible mechanisms that need to be taken into account: (i) PS-341-induced inhibition of Bcr/Abl expression may result directly in the decrease of IκB-α phosphorylation observed; (ii) in contrast, PS-341 was shown to inhibit the proteasome degradation of the IκB-α protein, which may sequester NF-κB in the cytoplasm. Moreover, the regulation of the level of DNA binding activity of NF-κB factors may be complex and may involve the relative abundance of NF-κB transactivating proteins. In fact, it has been reported that while Bcr-Abl may facilitate the nuclear translocation of NF-κB, it may also exert its action...
by increasing the transactivation function of ReIA/p65, or by increasing the stability of the p65 protein. While IM represents the treatment of choice for Bcr/Abl-positive diseases, some such diseases are becoming clinically resistant to this drug. We, therefore, explored the effects of PS-341 in combination with IM in IM-sensitive and IM-resistant cell lines. The results of our in vitro study suggest an important dose-schedule effect. The sequential combination of PS-341 and IM demonstrated synergistic pro-apoptotic effects in IM-sensitive cells. In contrast, antagonistic effects were observed when the cells were simultaneously exposed to the two drugs. An earlier study in multiple myeloma cells showed that PS-341 down-regulates expression of several growth/survival and anti-apoptotic signaling molecules and induces multiple apoptotic pathways. We have shown that PS-341 had only a rapid and transient inhibitory effect on NF-κB DNA binding activity, while inhibition of Bcr/Abl expression and phosphorylation was dose- and time-dependent, and correlated with the decrease in Bcl-XL expression. We think that exposure of cells to PS-341 for a short period, followed by treatment with IM, allows the two drugs to act similarly (but through different mechanisms) on several target signaling molecules. Simultaneous exposure to the two drugs may have opposite effects on the same kinase activity; however, this hypothesis requires experimental verification. In KBM-5R cells, pre-exposure to PS-341 did not sensitize the cells to IM, suggesting that proteasome inhibition does not alter and/or modify in vitro-acquired mechanisms of resistance to IM.

In summary, PS-341, as a single agent, is highly effective in inhibiting cell growth and inducing apoptosis in Bcr/Abl-positive cell lines both sensitive and resistant to IM. The emerging problem of clinical resistance to IM should encourage the development of treatment strategies including the exploration of new effective and well tolerated agents, such as PS-341. The results of our in vitro studies imply that caution should be exercised when designing clinical trials using combinations of PS-341 and IM.

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Pre-publication Report & Outcomes of Peer Review

Contributions
SG: led the study conduction from the beginning, performing most of the laboratory experiments, and wrote the draft of the manuscript; MB: constantly provided the guidelines and the required criticisms to the step by step study performance of the study and manuscript writing. All other authors listed in this manuscript contributed substantially to the study and gave final approval to the version to be pub-
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In the following paragraphs, Dr. Dazzi summarizes the peer-review process and its outcomes.

What is already known on this topic
Resistance to imatinib mesylate is becoming an emerging clinical problem in the treatment of chron-
ic myeloid leukemia (CML). New therapeutic agents are being developed. PS-341 is a new proteasome inhibitor that is cytotoxic in vitro on a wide range of tumor cells. It is known that PS-341 interferes with the regulation of cell cycle progression, and induces apoptosis. Its effect on BCR/ABL-positive cells has not been elucidated.

What this study adds
This study represents the first investigation on the effect of PS-341 in CML. It demonstrates that PS-341 potently suppresses growth and induces apoptosis of both imatinib sensitive and resistant cell lines. The authors suggest a strategy for sequential utilization of PS-341 and imatinib in a view of enhancing therapeutic efficacy and avoiding the development of imatinib resistance.