Proteasome Inhibition Leads to Significant Reduction of Bcr-Abl Expression and Subsequent Induction of Apoptosis in K562 Human Chronic Myelogenous Leukemia Cells

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ABSTRACT

The chimeric oncogene bcr-abl is detected in virtually every case of chronic myelogenous leukemia. It has been shown that cells (such as K562) expressing Bcr-Abl/p210, a protein tyrosine kinase, not only undergo cellular transformation but also demonstrate multiple drug resistance. Recent studies also demonstrate that the proteasome is involved in the survival signaling pathway(s). In the current study, we tested the hypothesis that the proteasome might play a role in regulating Bcr-Abl function. We have demonstrated by using a variety of inhibitors that inhibition of the proteasome, but not of the cysteine protease, activity is able to activate the apoptotic cell death program in K562 cells. Proteasome inhibition-induced apoptosis is demonstrated by condensation and fragmentation of nuclei, appearance of an apoptotic population with sub-G1 DNA content, the internucleosomal fragmentation of DNA, and cleavage of poly(ADP-ribose) polymerase, and can be blocked by a specific caspase-3-like tetrapeptide inhibitor. Western blot analysis with specific antibodies to c-Abl and Bcr proteins show that treatment of K562 cells with a proteasome inhibitor results in significant reduction of Bcr-Abl protein expression, which occurs several hours before the onset of apoptotic execution. Levels of c-Abl/p145 and Bcr/p160 proteins, however, remain essentially unaltered at that time. Furthermore, reduced Bcr-Abl expression is reflected in significantly attenuated Bcr-Abl-mediated protein tyrosine phosphorylation. Taken together, these results indicate that proteasome inhibition is sufficient to inactivate Bcr-Abl function and subsequently activate the apoptotic death program in cells that are resistant to apoptosis induced by chemotherapy.

Apoptosis, or programmed cell death, is a highly regulated, complex cellular process occurring in two physiological stages, commitment and execution (Earnshaw, 1995). The molecular events involved in the apoptotic commitment are not clearly understood. However, it has been found that the execution stage of apoptosis is initiated by activation of members of a specific cysteine protease family, named caspsases, which cleave specific intracellular proteins such as poly-(ADP-ribose) polymerase (PARP) (Martin and Green, 1995). These molecular events lead to morphological changes that are characteristic of apoptosis, including plasma membrane blebbing, cytoplasmic and nuclear condensation, nuclear fragmentation, and cellular fragmentation into apoptotic bodies (Earnshaw, 1995).

Chronic myelogenous leukemia (CML) is characterized by a translocation that creates a fusion between the bcr gene on chromosome 22 and the c-abl gene on chromosome 9 to form what is known as the Philadelphia t(9;22) chromosome (Ph1) (Mes-Masson et al., 1986). This translocation produces a chimeric oncogene, bcr-abl, which encodes a 210-kDa fusion protein (Bcr-Abl) with unregulated tyrosine kinase activity (Konopka et al., 1984). The tyrosine kinase activity of Bcr-Abl, which is the principal driving force behind its oncogenic potential, is responsible for mediating tyrosine phosphorylation of specific cellular proteins and Bcr-Abl itself (Lugo et al., 1990).

Although there is some evidence that the Bcr-Abl oncoprotein acts as a proliferative activator (Daley et al., 1990; Elefanty et al., 1990; Skorski et al., 1996), its major function appears to be to act as an apoptotic suppressor. This idea is

ABBREVIATIONS: PARP, poly(ADP-ribose) polymerase; CML, chronic myelogenous leukemia; LLnV, N-carbobenzoxy-L-leucyl-L-leucyl-norvaline; LLnL, N-acetyl-L-leucyl-L-leucyl-norleucinal; LLL, N-carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; LLM, N-acetyl-L-leucyl-L-leucyl-L-methioninal; VP-16, etoposide; E-64d, (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methyl-butanethoxy ethyl ester; DMSO, dimethyl sulfoxide; DEVD-FMK, acetyl-DEVD-fluoromethyl ketone.
supported by the following evidence. First, in vivo studies have demonstrated that massive clonal expansion of myeloid precursor cells in CML patients is due to an increased survival ability, but not to an increased proliferative rate (Koeffler and Golde, 1981). Indeed, primary Ph\(^1\)-positive leukemic cells expressing Bcr-Abl demonstrate an increased resistance to apoptosis induced by serum deprivation, irradiation, and chemotherapeutic agents (Bedi et al., 1994; Nishii et al., 1996). Second, transfection of the bcr-abl gene into Ba/F3 cells endues these cells with the drug-resistance phenotype (Nishii et al., 1996) and protects them against the apoptotic effects of growth factor withdrawal (Bedi et al., 1994). Third, when the Ber-Abl level in K562 cells was decreased by using its antisense oligonucleotides, cell growth was reduced due to an increased apoptosis, but not due to decreased DNA synthesis; the treated K562 cells had also lost their multidrug-resistant phenotype and became sensitized to drug-induced programmed cell death (McGahon et al., 1994; Rowley et al., 1996).

The 26S proteasome, composed of a 20S catalytic core and two associated 700-kDa regulatory proteins, is a large multicatalytic protease demonstrating at least trypsin-like, chymotrypsin-like, and peptidylglycine alpha-amidating carboxypeptidase activities (Hochstrasser, 1995). Chains of polyubiquitin, covalently linked to e-amino groups of lysine residues, mark a protein to be targeted for the proteasome for hydrolysis. Several cell cycle and apoptosis regulatory proteins, including cyclins, cyclin-dependent kinase inhibitors, tumor suppressor p53, and transcription factors E2F and nuclear factor Kappa B, are regulated through proteolysis via the ubiquitin/proteasome pathway (Hopkin, 1997).

Recently, studies using selective inhibitors of the proteasome have provided direct evidence that indicates that the proteasome functions both in promoting apoptosis and in protecting cells against apoptosis. These proteasome inhibitors include lactacystin, a highly specific inhibitor of the 20S proteasome (Fenteany et al., 1995), and tripeptide aldehydes, inhibitors of the proteasome chymotrypsin-like activity (Rock et al., 1994). It has been found that these proteasome inhibitors block the apoptotic process in thymocytes (Grimm et al., 1996) and neurons (Sadoul et al., 1996). In contrast, the same proteasome inhibitors induce apoptosis in human or mouse leukemia (Imajoh-Ohmi et al., 1995; Shinohara et al., 1996; Drexler, 1997) and other proliferating cell lines (Lopes et al., 1997). By using novel dipeptidyl proteasome inhibitors, we also found that inhibition of the proteasome activity is sufficient to rapidly induce apoptosis in human Jurkat T cells overexpressing Bel-2 and also in all human prostate, breast, tongue, and brain tumor cell lines tested (An et al., 1998). Furthermore, dipepidyl proteasome inhibitors selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in simian virus 40-transformed fibroblasts, but not in the parental normal, human fibroblasts (An et al., 1998). In the current study, we have investigated the ability of proteasome inhibitors, including tripeptide aldehydes and lactacystin, to induce apoptosis in K562 human chronic myelogenous leukemic cells, which express Bcr-Abl and are multidrug-resistant (Martin et al., 1990; McGahon et al., 1994; Rowley et al., 1996). We report here that these proteasome inhibitors first decrease the levels and tyrosine kinase activity of the Bcr-Abl protein and, subsequently, activate the apoptotic death program in these cells.

### Experimental Procedures

#### Materials
N-carbobenzyoxy-L-leucyl-L-leucyl-norvalinal (LLnV), \( N\)-acetyl-L-leucyl-L-leucyl-norleucinal (LLnL), \( N\)-carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (LLL), \( N\)-acetyl-L-leucyl-L-leucyl-L-methioninal (LLM), etoposide (VP-16), (25,35)-trans-epoxysuccinyl-L-leucylalami-do-3-methyl-butanylethyl ester (E-64d), dimethyl sulfoxide (DMSO), antipain, iodoacetamide, tosyl-L-lysine chloromethyl ketone, tosyl-L-phenylalanine chloromethyl ketone, and phenylmethyl-sulfonyl fluoride were purchased from Sigma Chemical Co. (St. Louis, MO). Lactacystin was obtained from Calbiochem (La Jolla, CA) and acetyl-DEVD-fluoromethyl ketone (DEVD-FMK) was obtained from Kamiya Biomedical Company (Seattle, WA). Stocks of LLnV, LLnL, LLL, LLM, lactacystin, VP-16, and DEVD-FMK were prepared by dissolving in DMSO such that the final concentration of the solvent in the medium did not exceed 0.1%. Purified mouse monoclonal antibody to caspase-3 (CPP32) was obtained from Transduction Laboratories (Lexington, KY); to PARP from Unité de Santé Environnement (Québec, Canada), and to c-Abl (Ab-3; derived from a fusion protein corresponding to the carboxyl region of the v-abl protein) from Oncogene Research Products (Cambridge, MA). Rabbit polyclonal antibody to Bcr, recognizing the peptide sequence corresponding to amino acids 2 to 21 of human Bcr, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and to ubiquitin from Sigma. Horseradish peroxidase-conjugated anti-phosphotyrosine antibody was from Transduction Laboratories (Lexington, KY).

#### Cell Culture and Treatment with Proteasome Inhibitors
K562 human CML cells were maintained in RPMI 1640 medium containing 10% fetal calf serum (Sigma), 100 U/ml penicillin, 100 \( \mu \)g/ml streptomycin, and 3 mM glutamine (growth medium) in a 5% CO\(_2\) atmosphere at 37°C. K562 cells were treated with a proteasome inhibitor at a concentration for the length of time noted in the legends to Figs. 1 to 8.

#### Nuclear Staining, Flow Cytometry, and DNA Fragmentation Assays
To assay nuclear morphology, K562 cells were washed with PBS, fixed with 70% ethanol, and stained with Hoechst 33258 (1 mM) for 30 min. The nuclear morphology of cells was visualized by a fluorescence microscope (Olympus BH2; Olympus Optical Co., LTD, Tokyo, Japan). DNA content analysis by flow cytometry was performed as described previously (Nicoletti et al., 1991). Briefly, K562 cells were fixed with 70% ethanol, stained with propidium iodide (50 \( \mu \)g/ml) for 30 min at room temperature, and analyzed immediately in a flow cytometer. DNA fragmentation was analyzed as described (Grant et al., 1992). At each time point, K562 cells were washed in PBS and resuspended in 0.7 ml of a buffer containing 10 mM Tris-HCl, 10 mM EDTA, 0.5% SDS, and 200 \( \mu \)g/ml protease K. The cell mixtures were incubated at 5°C for 2 h and then treated with 25 \( \mu \)g/ml RNase at 37°C for 1 h. After incubation, DNA was precipitated with 1.5 volume of ethanol and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The prepared DNA samples were analyzed in a 2% agarose gel containing 0.1% SDS, followed by staining with ethidium bromide.

#### Whole-Cell Extracts and Western Blot Assay
Whole-cell extracts and the enhanced chemiluminescence Western blot assay were performed as described previously (An and Dou, 1996). Each lane contains an equal amount of protein (40–70 \( \mu \)g).

### Results

#### Tripeptidyl Aldehyde LLnV Activates Caspase Cascade and Induces Apoptosis in K562 Cells
By using different proteasome inhibitors, several groups have demonstrated that the proteasome is involved in the survival-signaling pathway(s) (Imajoh-Ohmi et al., 1995; Shinohara et al., 1996; Drexler, 1997; Lopes et al., 1997; An et al., 1998). Because the Bcr-Abl oncprotein functions as a proliferative...
activator (Daley et al., 1990; Elefanty et al., 1990; Skorski et al., 1996) as well as an apoptotic suppressor (Bedi et al., 1994; Nishii et al., 1996), we hypothesized that the proteasome might play a role in the regulation of Bcr-Abl function and that inhibition of the proteasome activity might overcome Bcr-Abl-mediated drug-resistance and induce apoptosis in Bcr-Abl-expressing cells. To test this hypothesis, we used K562 human CML cells that express Bcr-Abl (Martin et al., 1990). When K562 cells were treated for 24 to 48 h with 50 \( \mu M \) LLnV, a tripeptidyl aldehyde that can block the proteasome activity effectively (Rock et al., 1994), apoptosis indeed occurred, as demonstrated by condensation and fragmentation of nuclei (Fig. 1A), appearance of an apoptotic population with sub-G1 DNA content (Fig. 1B, indicated by Ap), and the internucleosomal fragmentation of DNA (Fig. 1C, DNA ladders). Exposure of K562 cells to LLnV for 24 h also induced the processing of caspase-3/CPP32 (data not shown), which is required for its activation (Martin and Green, 1995), and complete cleavage of PARP to a p85 fragment (Fig. 1D, lane 6 versus lane 1). None of these LLnV-induced events were observed in K562 cells treated with the solvent DMSO (Fig. 1), demonstrating drug specificity. Because K562 cells lack functional p53 protein (Shao et al., 1996), LLnV-induced apoptosis in these cells is p53-independent.

To provide additional evidence for the involvement of caspase activation in LLnV-induced apoptosis in K562 cells, we used DEVD-FMK, a tetrapeptide inhibitor that preferentially inhibits caspase-3-like activities (Nicholson et al., 1995). When incubated with LLnV-treated K562 cells, DEVD-FMK effectively prevented, in a concentration-

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**Fig. 1.** Tripeptide aldehyde LLnV induces apoptosis in K562 human chronic myelogenous leukemia cells. A, nuclear staining assay. K562 cells were treated with either 50 \( \mu M \) LLnV or its solvent DMSO for 24 h, followed by measurement of apoptotic nuclear changes, as described in Materials and Methods. The percentage of nuclei demonstrating apoptotic condensation and fragmentation at the indicated condition varied somewhat between experiments (60–95%) and averaged 79.9% (\( n = 4 \)). The percentage of apoptotic nuclei from LLnV-treated cells in this experiment was 92%. B, flow cytometry. Exponentially grown K562 cells (0 h) were treated with 50 \( \mu M \) LLnV for 48 h, followed by measurement of sub-G1 (indicated by Ap), G1, S, and G2-M cell populations (see Materials and Methods). The units of the y-axis represent cell numbers (in total 10,000 events) and those of the x-axis represent cellular DNA content. The region of M1 represents sub-G1 DNA content of apoptotic cell population (Ap), which was increased significantly when K562 cells were treated with LLnV (compare the right side to the left side). The regions of M2, M3, and M4 represent DNA content of G1, S, and G2-M phase cells, respectively. C, DNA fragmentation assay. Exponentially grown K562 cells (0 h) were treated with 50 \( \mu M \) LLnV for up to 48 h, followed by measurement of apoptotic DNA ladder formation. The molecular masses of DNA fragments detected in lane 3 are about 180 bp, 360 bp, . . . , respectively (from bottom to top), based on the migration positions of the used DNA 123-bp ladder markers (D5042, Sigma). D, PARP cleavage assay. Exponentially grown K562 cells (0 h) were treated with either 50 \( \mu M \) LLnV or its solvent DMSO (indicated by D) for 4, 8, 12, or 24 h, followed by measurement of PARP cleavage. The molecular mass of intact PARP protein is 116 kDa and that of the apoptosis-associated cleavage fragment of PARP is 85 kDa (p85/PARP).
dependent manner, induction of apoptotic nuclear changes: ~70% inhibition at 30 μM and >90% at 100 μM (Fig. 2A). Consistent with that, inhibition of PARP cleavage by DEVD-FMK was also concentration-dependent: ~75% at 30 μM and 90 to 100% at 100 or 300 μM (Fig. 2B, lanes 3–5 versus lane 2). These data demonstrate that LLnV-induced apoptosis in K562 cells requires activation of caspases.

Inhibition of Activity of the Proteasome, but Not Cysteine Proteases, Is Responsible for Induction of Apoptosis in K562 Cells. The tripeptidyl aldehydes that were originally developed as inhibitors of the cysteine proteases (such as calpain I and cathepsin B) also inhibit the proteasomal activity (Rock et al., 1994). However, LLnV and LLnL were 50- to 800-fold more potent than LLM in inhibition of the purified 20S or 26S proteasome activity, whereas the three aldehydes inhibit calpain and cathepsin with similar potencies (Rock et al., 1994). To demonstrate that induction of apoptosis in K562 cells is due to blockade of activity of the proteasome but not of the cysteine proteases, the following experiments were performed. First, K562 cells were treated for 18 h with a variety of tripeptidyl aldehydes at a fixed concentration (50 μM), followed by measurement of apoptosis-specific PARP cleavage. LLnV and LLnL induced an almost complete cleavage of PARP, whereas LLL had a partial effect. In contrast, LLM was completely inactive at inducing PARP cleavage (Fig. 3A, lanes 2–5). The rank of the apoptosis-inducing activity for these inhibitors in K562 cells, therefore, was LLnV = LLnL > LLL >> LLM. Treatment of K562 cells with the DNA-damage agent VP-16 failed to induce the process of PARP cleavage (Fig. 3A, lane 6), confirming that K562 cells are resistant to this drug (Bedi et al., 1994; Nishii et al., 1996).

K562 cells were also treated with various concentrations of LLnV and LLM, and the effects were compared. Treatment with 10 to 30 μM LLnV for 20 h caused a complete cleavage of PARP (Fig. 3B, lanes 6 and 7 versus 1). In addition, a treatment with 3 μM LLnV for 42 h was also able to induce ~20% PARP cleavage (Fig. 3C, lane 4 versus lanes 1 and 2). In contrast, LLM did not induce PARP cleavage even when used at up to 100 μM for 42 h (Fig. 3C, lanes 5–9), although at 300 μM for 42 h, it caused ~5% PARP cleavage (Fig. 3C, lane 10). Therefore, the potency for LLnV to induce PARP cleavage is at least 100-fold higher than that of LLM, which...
corresponds to the abilities of these two compounds to inhibit the activity of the isolated proteasome but not calpain and cathepsin B (Rock et al., 1994).

To provide direct evidence that induction of apoptosis in K562 cells is due to inhibition of the proteasome but not cysteine proteases, we used more specific inhibitors of the proteasome and cysteine proteases. Lactacystin, a microbial metabolite, specifically inhibits the MB1 subunit of the proteasome (Fenteany et al., 1995) and is structurally distinct from tripeptide aldehyde proteasome inhibitors. Furthermore, lactacystin has no or little effect in the inhibition of the activity of a purified calpain (Mellgren, 1997). When K562 cells were treated with 10 μM lactacystin for 24 to 48 h, apoptosis occurred, as evidenced by specific morphological changes in the nuclei (Fig. 4A versus the control in Fig. 1A), an increase in the level of apoptosis-associated sub-G1 cell population (indicated by Ap, Fig. 4B versus the control in Fig. 1B), and the internucleosomal fragmentation of DNA (Fig. 4C). A 24-h treatment also caused cleavage of PARP to the p85 fragment (Fig. 4D, lane 3). By contrast, the specific cysteine protease inhibitor E-64d (McGowan et al., 1989), when used at 50 μM for up to 32 h, did not induce either apoptotic nuclear changes (Fig. 5A) or PARP cleavage (Fig. 5B).

Finally, inhibition of the proteasome, but not cysteine protease, activity should cause increased levels of polyubiquitinated proteins. Indeed, when lysates of LLnV-treated K562 cells were immunoblotted with an antiserum to ubiquitin, accumulation of high molecular weight polyubiquitinated proteins was detected (Fig. 6, lanes 2–6 versus lane 1). In fact, the polyubiquitinated proteins were induced to maximum levels at as early as 1 h after the addition of LLnV (Fig. 6, lane 2). Taken together, our results demonstrate that inhibition of the proteasome, but not of a cysteine protease, activity is responsible for induction of apoptosis in K562 cells.

**Inhibition of the Proteasome Activity in K562 Cells Leads to a Dramatic Decrease of Bcr-Abl Expression Before Induction of Apoptosis.** Because the multidrug-resistance phenotype of K562 cells is conferred by the chimeric Bcr-Abl oncoprotein (McGahan et al., 1994; Rowley et al., 1996), we hypothesized that inactivation of Bcr-Abl function should have occurred before apoptosis induction in K562 cells after the treatment with proteasome inhibitors. To test this hypothesis, we measured levels of Bcr-Abl protein in the experiments described above (Figs. 1–5).

The level of Bcr-Abl/p210 protein expression was high in exponentially growing K562 cells (Fig. 7A, lane 1), as detected by immunoblotting with an anti-c-Abl antibody (derived from a fusion protein corresponding to the carboxyl region of the v-abl protein). However, the Bcr-Abl protein expression was slightly decreased after treatment with 50 μM LLnV for 4 h (Fig. 7A, lane 3 versus lanes 1–2). A significant reduction in Bcr-Abl protein expression was observed after 8 h of treatment (Fig. 7A, lane 4). However, no or little change in expression of c-Abl/p145 protein was observed in the same samples (Fig. 7A, lanes 3 and 4 versus lane 2). When similar lysates (from K562 cells treated with 50 μM LLnV for 7 h) were immunoblotted with an anti-Bcr antibody (recognizing its amino terminal sequence), significant reduction in Bcr-Abl expression was again observed, whereas the level of Bcr/p160 was only slightly reduced (Fig. 7B, lane 2 versus lane 1). In the control K562 cell lysate, the anti-Bcr antibody also detected a band of 130 kDa (the fastest-migrating abundant band in lane 1), which might represent Bcr/p130 that was previously reported to be expressed in K562 and other cell lines (Dhut et al., 1990). Expression of this protein was nearly completely lost after 7 h of LLnV treatment (Fig. 7B, lane 2 versus lane 1). More importantly, after 7 or 8 h of treatment, no apoptotic changes such as PARP cleavage had been observed (see Fig. 1D, lane 4). The level of Bcr-Abl protein expression remained low after 12 h of treatment, and further decreased after 24 h (Fig. 7A, lanes 5 and 6 versus lane 7). Only after 24 h of treatment was the level of Abl protein expression notably decreased (Fig. 7A, lane 6 versus lane 7). The process of PARP cleavage started at 12 h of treatment and completed at 24 h of treatment (Fig. 1D, lanes 5–6). These results demonstrate that LLnV treatment of K562 cells leads to a significant reduction of Bcr-Abl protein expression and subsequent induction of apoptosis. Neither reduction of Bcr-Abl expression nor induction of apoptosis was seen in K562 cells treated with DMSO (Figs. 1 and 7A), demonstrating that apoptosis-associated Bcr-Abl reduction is drug-specific.

We then determined the levels of Bcr-Abl protein expression in the concentration-response experiment with LLnV and LLM. A 42-h treatment with 1 μM LLnV neither decreased the Bcr-Abl protein level (Fig. 7C, lane 3 versus lane 2) nor produced the p85/PARP fragment (Fig. 3C, lane 3). However, LLnV at 3 μM significantly reduced the level of Bcr-Abl (Fig. 7C, lane 4) and also induced PARP cleavage (Fig. 3C, lane 4). Further increase in the concentration of LLnV resulted in a complete loss of Bcr-Abl protein expression (Fig. 7C, lanes 5 and 6) and a complete cleavage of PARP (data not shown but see Fig. 3B, lanes 6 and 7). In contrast, LLM, only when used at 300 μM for 42 h, was able to decrease the level of Bcr-Abl protein expression (Fig. 7C, lane 12 versus lanes 7–11) and induce cleavage of PARP (Fig. 3C, lane 10 versus lanes 5–9). Figure 7C also indicates that the ability of LLnV to reduce the Bcr-Abl protein expression is about 100-fold more potent than that of LLM (compare lane 4 versus lane 12), which corresponded exactly to their potencies in induction of PARP cleavage (Fig. 3C).

Furthermore, treatment of K562 cells with the specific proteasome inhibitor lactacystin for 8 h also reduced the level of Bcr-Abl, but not of Abl, protein expression (Fig. 7D, lane 2 versus lane 1). At this time, PARP cleavage had not occurred (Fig. 4D, lane 2). A 24-h treatment with lactacystin further decreased the level of Bcr-Abl (and Abl) protein (Fig. 7D, lane 3) and also induced PARP cleavage (Fig. 4D, lane 3). By contrast, treatment with the specific cysteine protease inhibitor E-64d at 50 μM for up to 32 h neither reduced Bcr-Abl protein level (Fig. 7E) nor induced apoptosis (Fig. 5). It should be noted that lactacystin (IC50 = 1 μM; Mellgren, 1997) is less potent than LLnV (IC50 = 21 nM; Rock et al., 1994) in inhibiting the activity of a purified proteasome, although lactacystin is more specific for the inhibition of the proteasome activity. Their different potencies in inhibiting the proteasome activity may be responsible for their different abilities in reducing Bcr-Abl expression and killing K562 cells (Figs. 1, 3, 4, and 7). Regardless of that, our data strongly suggest that inhibition of the proteasome activity in K562 cells first leads to reduction of Bcr-Abl protein expression and, subsequently, induction of apoptosis.

To establish that the decreased Bcr-Abl expression leads to...
Fig. 4. The specific proteasome inhibitor lactacystin induces apoptosis in K562 cells. A, nuclear staining assay. K562 cells were treated with 10 μM lactacystin (Lact) for 24 h, followed by measurement of apoptotic nuclear changes, as described in Materials and Methods. The percentage of apoptotic nuclei in this experiment was 75%. Similar results were obtained in three independent experiments. B, flow cytometry. K562 cells were treated with 10 μM lactacystin for 48 h, followed by measurement of DNA content by flow cytometry (see Materials and Methods). The units of the y-axis represent cell numbers (in total 10,000 events) and those of the x-axis represent cellular DNA content. The regions of M1, M2, M3, and M4 represent DNA content of sub-G1 (indicated by Ap), G1, S, and G2-M phase cells, respectively. The Ap cell population was increased significantly when K562 cells were
attenuation of Bcr-Abl-mediated protein tyrosine phosphorylation, the lysates, which were prepared from K562 cells treated with 50 $\mu$M LLnV for 7 h (identical with those in Fig. 7B), were also analyzed by immunoblotting with an anti-phosphotyrosine antibody. As shown in Fig. 8, several heavily tyrosine-phosphorylated proteins were observed in control K562 cell lysates (lane 1), which is typical of Bcr-Abl-expressing cells (Okuda et al., 1996). A band of ~210 kDa is Bcr-Abl itself (indicated in lane 1) because Bcr-Abl undergoes autophosphorylation on tyrosine residues (Lugo et al., 1990) and because this band was not detected in an HL-60 cell lysate (data not shown). After 7 h of LLnV treatment, the levels of tyrosine phosphorylation for nearly all of the major phosphotyrosine-containing proteins, as well as Bcr-Abl itself, were significantly reduced (Fig. 8, lane 2 versus lane 1). This demonstrates that LLnV-mediated reduction of Bcr-Abl protein expression in K562 cells leads to inactivation of Bcr-Abl tyrosine kinase function, which is responsible for the decrease in the level of Bcr-Abl-mediated protein tyrosine phosphorylation before the initiation of apoptotic execution.

To determine whether proteasome inhibition causes proteolytic degradation of Bcr-Abl protein, a variety of protease inhibitors were tested for their ability to inhibit this event, including DEVD-FMK, antipain, iodoacetamide, tosyl-L-lysine chloromethyl ketone, tosyl-L-phenylalanine chloromethyl ketone, phenylmethylsulfonyl fluoride, leupeptin, and dichloroisocoumarin. None of these protease inhibitors were able to block the LLnV-induced decrease in the Bcr-Abl protein levels under the experimental conditions (data not shown). It remains to be determined what molecular mechanisms are responsible for proteasome inhibition-mediated reduction of Bcr-Abl protein expression.

**Discussion**

The chimeric oncoprotein Bcr-Abl is responsible for conferring both transformation properties (Daley et al., 1990; Elefanty et al., 1990; Skorski et al., 1996) and the multiple drug-resistance phenotype (Bedi et al., 1994; Nishii et al., 1996) to human CML cells. In the current study, we have shown that the death-promoting activity of proteasome inhibitors is able to overcome the antiapoptotic function of Bcr-Abl in K562 cells (Figs. 1, 3, and 4) and that proteasome inhibitor-induced apoptosis in these cells is p53-independent and requires activation of the caspases (Fig. 2). In addition, we have found that before induction of apoptosis, proteasome inhibition decreases the levels and tyrosine kinase activity of the Bcr-Abl protein (Figs. 7 and 8), suggesting a novel mechanism for inactivating the Bcr-Abl function.

The following evidence supports the hypothesis that inhibition of the proteasome, but not of the cysteine protease, activity is responsible for induction of apoptosis in K562 cells. First, LLnV, previously shown to be a potent proteasome inhibitor (compare to the DMSO-treated cells in Fig. 1A). C, DNA fragmentation assay. Exponentially grown K562 cells (0 h) were treated with 10 $\mu$M lactacystin for up to 48 h, followed by measurement of apoptotic DNA ladder formation. The molecular masses of DNA fragments detected in lane 3 are about 180 bp, 360 bp, . . . , respectively (from bottom to top), based on the migration positions of the used DNA 123-bp ladder markers. D, PARP cleavage assay. Exponentially grown K562 cells (0 h) were treated with 10 $\mu$M lactacystin for up to 24 h, followed by measurement of PARP cleavage. The molecular masses of intact PARP protein and its cleavage fragment (p85/PARP) are 116 and 85 kDa, respectively.
inhibitor in vitro and in vivo (Rock et al., 1994), effectively induces apoptosis in K562 cells (Figs. 1 and 3). Other structurally similar peptide aldehydes (LLnL and LLL), which also inhibit the proteasome in vitro and in vivo (Rock et al., 1994), also induce apoptosis-associated PARP cleavage in K562 cells (Fig. 3A). In contrast, LLM, an inhibitor of the cysteine proteases but only a weak inhibitor of the proteasome (Rock et al., 1994), does not activate the caspase cascade at even a very high concentration (Fig. 3C). The relative potencies of LLnV to LLM for inducing apoptosis correspond to the abilities of these two compounds to inhibit the activity of the isolated proteasome but not calpain and cathepsin B (Rock et al., 1994). Second, the microbial metabolite lactacystin, a specific proteasome inhibitor which is structurally distant from tripeptide aldehydes (Fenteany et al., 1995), is also able to induce apoptosis-associated PARP cleavage in K562 cells (Fig. 3A). In contrast, LLM at up to 100 μM neither decreased the level of Bcr-Abl protein (data not shown) nor induced apoptosis (Fig. 3C). Furthermore, the relative potencies of LLnV to LLM for reducing the level of Bcr-Abl oncoprotein match exactly those of these two compounds for inducing apoptosis (Figs. 3C and 3C), which also correspond to their inhibitory activities toward the isolated proteasome but not calpain and cathepsin B (Rock et al., 1994). Third, treatment of K562 with the specific proteasome inhibitor lactacystin reduced the level of Bcr-Abl protein at 8 h (Fig. 7D) and induced apoptosis at 24 h (Fig. 4D). In contrast, the specific cysteine protease inhibitor strongly suggest that inhibition of the proteasome activity in K562 cells leads to activation of the apoptotic death program.

Because expression of Bcr-Abl oncoprotein in K562 cells confers their drug-resistance phenotype (Bedi et al., 1994; Nishii et al., 1996; Fig. 3A, lane 6), we hypothesized that proteasome inhibition must have caused inactivation of the Bcr-Abl oncoprotein before apoptosis induction in K562 cells. The following evidence supports this hypothesis. First, treatment of K562 cells with the tripeptidyl proteasome inhibitor LLnV caused a significant reduction of the Bcr-Abl protein expression between 4 and 8 h of treatment (Fig. 7A), which was followed by induction of apoptosis between 12 and 24 h (Fig. 1D). Therefore, the level of the Bcr-Abl oncoprotein was decreased at least 4 h before apoptosis was detected. The Bcr-Abl reduction can be detected by specific antibodies to either c-Abl or Bcr protein (Fig. 7, A and B). Because no notable changes in the levels of c-Abl/p145 and Bcr/p160 were detected at up to 8 h of treatment (Fig. 7, A and B), the reduction of Bcr-Abl by proteasome inhibition seems selective. Second, we have found that LLnL and LLL also caused a decrease in the levels of Bcr-Abl protein at 8 h (Fig. 7D) and induced apoptosis at 24 h (Fig. 4D). In contrast, treatment of K562 with the specific proteasome inhibitor lactacystin reduced the level of Bcr-Abl protein at 8 h (Fig. 7D) and induced apoptosis at 24 h (Fig. 4D). In contrast, the specific cysteine protease inhibitor
E-64 days did not induce either of these two events even when used at a high concentration for a long period of time (Figs. 7E and 5). Fourth, the decreased level of Bcr-Abl oncoprotein is directly associated with the decreased levels of tyrosine phosphorylation for nearly all of the major phosphorytrosine-containing proteins including Bcr-Abl itself (Figs. 7, 8), indicating inactivation of Bcr-Abl tyrosine kinase activity. Fifth, LNV induces a rapid accumulation of high molecular weight polyubiquitinated proteins in K562 cells to maximum levels within 1 h of treatment (Fig. 6), which occurred before reduction of Bcr-Abl expression (between 4 and 8 h; Fig. 7A). Therefore, it appears that inhibition of the proteasome first promotes the removal of the antiapoptotic activity of Bcr-Abl and subsequently induces programmed cell death in K562 cells.

The following arguments suggest that inactivation of Bcr-Abl function by proteasome inhibition is essential for induction of apoptosis in K562 cells. First, overexpression of the Bcr-Abl oncoprotein inhibits apoptosis induced by multiple stimuli (Bedi et al., 1994; Nishii et al., 1996). Second, treatment of K562 cells with antisense oligonucleotides targeting Bcr-Abl decreases Bcr-Abl protein expression and sensitizes the cells to drug-induced programmed cell death (McGahan et al., 1994; Rowley et al., 1996). Third, our results from kinetics experiments (Figs. 1, 4, 7, and 8) demonstrate that the levels and tyrosine kinase activity of Bcr-Abl are decreased before K562 apoptotic cell death induced by proteasome inhibitors.

It remains unclear how the decrease in the levels and tyrosine kinase activity of Bcr-Abl by proteasome inhibition triggers K562 cell apoptosis. We propose that reduction of the Bcr-Abl protein expression and consequent inactivation of the Bcr-Abl function could be involved in committing a K562 cell to undergoing apoptotic death. Because specific targeting of Bcr-Abl protein is not sufficient for induction of apoptosis (McGahan et al., 1994; Rowley et al., 1996), a proteasome inhibitor, a single agent, therefore, should have dual functions: inactivation of Bcr-Abl and induction of apoptosis. It is possible that these two functions of the proteasome inhibitor work together to trigger K562 cellular apoptosis. This hypothesis is supported by the following arguments. It has been reported that Bcr-Abl-mediated protection is regulated by Bel-2 (Sanchez-Garcia and Grutz, 1995) or Bel-XL (Amarante-Mendes et al., 1998a), two apoptosis inhibitors, proteins that can be overexpressed by overexpression of Bax (Kobayashi et al., 1998), another Bel-2 family protein and an apoptosis inducer. Furthermore, the Bel-2 family proteins are involved in regulating the proapoptotic mitochondrial release of cytochrome c into cytosol, which activates the caspase-3 pathway involved in the execution of apoptosis (Green and Reed, 1998). Indeed, overexpression of Bcr-Abl blocks release of cytochrome c from mitochondria to the cytosol (Martins et al., 1997; Amarante-Mendes et al., 1998b). We also found that when K562 cells were treated with a proteasome inhibitor, cytochrome c was released into cytosol immediately after the reduction of the Bcr-Abl protein expression (B. Li, K. Morrow, and Q.P.D., unpublished data). We are currently investigating the detailed molecular mechanisms for the link between reduction of Bcr-Abl protein expression and induction of apoptosis by inhibition of proteasome inhibition-induced decrease in the levels of Bcr-Abl oncoprotein remain unknown. We have found that it cannot be inhibited by a variety of the tested protease inhibitors (data not shown), which suggests that the Bcr-Abl level reduction, if due to proteolytic degradation, must be mediated by an unique protease. Alternatively, a different mechanism may be responsible. One possibility is that inhibition of the proteasome could lead to inhibition of Bcr-Abl transcription. This remains to be determined by future studies. Regardless of that, the results presented herein suggest that reduction of the Bcr-Abl oncoprotein is a novel mechanism by which proteasome inhibitors overcome multidrug-resistance that Bcr-Abl confers and induce p53-independent apoptosis. Because loss of functional p53 protein may play a role in the transition from the chronic phase of CML to the blast crisis in the latter stages of the disease (Skorski et al., 1996), proteasome inhibitors might have potential as chemotherapeutic agents for treating CML and related diseases.

References


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