Low-Level Expression of Proapoptotic Bcl-2–Interacting Mediator in Leukemic Cells in Patients with Chronic Myeloid Leukemia: Role of BCR/ABL, Characterization of Underlying Signaling Pathways, and Reexpression by Novel Pharmacologic Compounds

Karl J. Aichberger,1 Matthias Mayerhofer,1 Maria-Theresa Krauth,1 Anja Vales,1 Rudin Kondo,1 Sophia Derdak,2 Winfried F. Pickl,1 Edgar Selzer,3 Michael Deininger,5 Brian J. Druker,6 Christian Sillaber,1 Harald Esterbauer,2 and Peter Valent1

1Department of Internal Medicine I, Division of Hematology and Hemostaseology- the Center of Excellence in Clinical and Experimental Oncology; Institute of Immunology; Department of Radiation Therapy; Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Vienna, Austria; 2Oregon Health and Science University Cancer Institute, Center for Hematological Malignancies; and 3Howard Hughes Medical Institute, Portland, Oregon

Abstract
Chronic myeloid leukemia (CML) is a myeloproliferative disease in which BCR/ABL enhances survival of leukemic cells through modulation of proapoptotic and antiapoptotic molecules. Recent data suggest that proapoptotic Bcl-2–interacting mediator (Bim) plays a role as a tumor suppressor in myeloid cells, and that leukemic cells express only low amounts of this cell death activator. We here show that primary CML cells express significantly lower amounts of bim mRNA and Bim protein compared with normal cells. The BCR/ABL inhibitors imatinib and AMN107 were found to promote expression of Bim in CML cells. To provide direct evidence for the role of BCR/ABL in Bim modulation, we employed Ba/F3 cells with doxycycline-inducible expression of BCR/ABL and found that BCR/ABL decreases expression of bim mRNA and Bim protein in these cells. The BCR/ABL-induced decrease in expression of Bim was found to be a posttranscriptional event that depended on signaling through the mitogen-activated protein kinase pathway and was abrogated by the proteasome inhibitor MG132. Interestingly, MG132 up-regulated the expression of bim mRNA and Bim protein and suppressed the growth of Ba/F3 cells containing wild-type BCR/ABL or imatinib-resistant mutants of BCR/ABL. To show functional significance of “Bim reexpression,” a Bim-specific small interfering RNA was applied and found to rescue BCR/ABL-transformed leukemic cells from imatinib-induced cell death. In summary, our data identify BCR/ABL as a Bim suppressor in CML cells and suggest that reexpression of Bim by novel tyrosine kinase inhibitors, proteasome inhibition, or by targeting signaling pathways downstream of BCR/ABL may be an attractive therapeutic approach in imatinib-resistant CML. (Cancer Res 2005; 65(20): 9436–44)

Introduction
Chronic myeloid leukemia (CML) is a disorder of hematopoietic progenitor cells in which leukemic cells display the translocation t(9;22) and the resulting oncogene BCR/ABL (1–3). The respective oncoprotein, BCR/ABL, exhibits constitutive tyrosine kinase activity and activates a number of downstream signaling cascades including the Ras/Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) pathway, the phosphoinositide 3-kinase (PI3K) pathway, and the signal transducer and activator of transcription 5 pathway (4–8). In addition, BCR/ABL converts cytokine-dependent cell lines to growth factor independence (9).

Several different mechanisms are considered to contribute to BCR/ABL-dependent growth and accumulation of leukemic cells in patients with CML (10–12). One important mechanism seems to be inhibition of apoptosis (10, 11, 13–16). Thus, CML cells reportedly express a number of antiapoptotic molecules that may contribute to enhanced survival of leukemic cells (13–17). Likewise, it has been shown that CML cells express several members of the Bcl-2 family including Bcl-xL, Mcl-1, and Bcl-2 (13–18). However, the relative contribution of each of these molecules to inhibition of apoptosis in CML cells has not been clarified yet. In addition, some of these molecules may only be expressed in leukemic cells in a subgroup of patients (17). Moreover, the actual role and effects of Bcl-2 and of other antiapoptotic molecules may depend on the presence of endogenous inhibitors and antagonists that may be coexpressed in leukemic cells.

A major inhibitor and antagonist of Bcl-2 is the Bcl-2–interacting mediator (Bim), a BH3-only protein of the Bcl-2 family that is considered to have proapoptotic effects in various cells (19–21). Originally, Bim was described as a Bcl-2-binding protein that counteracts the antiapoptotic function of Bcl-2 and Bcl-xL in various cell lines (19).

More recently, the expression and function of Bim has been analyzed in the context of myeloid leukemias (22–24). With regard to CML, it was found that the BCR/ABL-positive cell line K562 expresses relatively low levels of Bim, and that incubation of these cells with the BCR/ABL tyrosine kinase inhibitor imatinib results in an increase in Bim expression (23, 24). Based on these data, it was hypothesized that Bim is regulated by BCR/ABL in CML cells (24). However, thus far, no direct proof for this hypothesis has been provided. In addition, although expression of Bim has been analyzed in CML-derived cell lines, there is no information about Bim expression in primary CML cells.

The aims of the present study were to examine the levels of Bim in primary CML cells and to define the actual role of BCR/ABL in abnormal expression of Bim in leukemic cells. In addition, we...
attempted to define signaling pathways underlying oncogene-dependent suppression of Bim in CML cells. We also examined whether Bim reexpression by pharmacologic compounds is associated with cell death and thus can be exploited as basis for the development of new therapeutic concepts.

Materials and Methods

Constructs. Plasmids used in this study were PE EE BimH, hygro, PET EE Bim, hygro, and PET EE Bim, hygro (ref. 19; kindly provided by David C.S. Huang, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). cDNAs were cloned into the pTRE vector (Invitrogen, Carlsbad, CA). The Bim reporter gene construct, Bim-Luc (ref. 25; kindly sent by Jonathan Gilley, Institute of Child Health, University College London, United Kingdom) consisted of a bim promoter fragment spanning 5.2 kb upstream of the transcriptional start site cloned into a pGL3-basic backbone (Promega, Madison, WI).

Isolation of primary cells. Primary leukemia cells were obtained from the peripheral blood or bone marrow of 10 patients with chronic-phase CML, three with accelerated phase, and two with blast phase CML. Normal bone marrow cells were obtained from lymphoma patients (routine staging) without bone marrow involvement (n = 11) and one with unexplained anemia. Informed consent was obtained in each case. Peripheral blood and bone marrow mononuclear cells were isolated using Ficoll. After isolation, cells were kept in RPMI 1640 medium (Mediatech Cellgro, Herndon, VA) and 10% FCS (Life Technologies, Carlsbad, CA) at 37°C in the presence or absence of granulocyte macrophage colony-stimulating factor (GM-CSF, 100 ng/mL) before drug exposure. In select experiments, cells were kept in interleukin-3 (IL-3, 100 ng/mL; PeproTech, Rocky Hill, NJ) before drug exposure. In select experiments, primary CML cells were transfected with Bim siRNA and luciferase siRNA using Lipofectin (Invitrogen; 10 μg/mL) as described by the supplier. K562 cells were incubated with 200 nmol/L bim siRNA or with 200 nmol/L luciferase siRNA at 37°C for 4 hours. Then, cells were incubated with control medium, imatinib (1 μmol/L) or MG132 (25 μmol/L) at 37°C for 24 hours. Thereafter, cells were subjected to Western blot analysis and determination of the percentage of apoptotic cells. In select experiments, primary CML cells were transfected with Bim siRNA and luciferase siRNA using Lipofectin (1 μg/mL) and were then exposed to control medium or imatinib (2 μmol/L) for 48 hours.

Lentiviral-mediated gene transfer of Bim into primary chronic myelogenous leukemia cells. The plasmids pWP7-GFP, pSPAX2, pMD2G, and pRSVvev were kindly provided by Didere Trono (Swiss Federal Institute of Technology, Lausanne, Switzerland). pWP7-GFP was modified by replacing GFP with an oligonucleotide containing a multicloning site (MCS) including a restriction site for BamHI (pWP7-MCS). Bim was amplified from KU812 cDNA using a proofreading polymerase (Pfu ultra, Stratagene, La Jolla, CA) and primer pairs containing BamHI restriction overhangs (underlined): Bim forward 5′-CGGGATCCGACGAGGAGGAAGAACCTTCTCGA-3′ and Bim reverse 5′-CGGGAACCTCAATGGAGGTTCCAGTTCCACA-3′. PCR-amplified Bim was cut by BamHI and cloned into pWP7-MCS (pWP7-Bim). Recombinant lentiviruses were produced by cotransfection of HEK-293FT cells with pSPAX2, pMD2G, pRSVvev, and either pWP7-Bim, or pWP7-GFP using LipofectAMINE 2000 (Invitrogen) according to published techniques (31). To improve viability, pWP7-Bim-transfected HEK-293FT cells were cotransfected with pcdNA3-bcl-xL (26). Primary CML cells (peripheral blood mononuclear cells from a patient with chronic-phase CML) were transduced with pWP7-Bim or pWP7-GFP (control). After infection, cells were maintained in RPMI 1640 containing 10% FCS and GM-CSF (100 ng/mL) for 10 days. On days 5, 7, and 10, cells were examined for expression of bim mRNA by RT-PCR and the percentage of apoptotic cells (cytospin preparations). cDNA synthesis was done using the Protocscript First-Strand cDNA synthesis kit (New England Biolabs, Beverly, MA) with 1 μg of total RNA in a volume of 50 μL. PCR conditions were 35 cycles of denaturation at 94°C (60 seconds), annealing at 58°C (40 seconds), and extension at 72°C (40 seconds) followed by terminal extension at 72°C (10 minutes). Primer sequences were Bim forward 5′-ATGCCAAGAACCTTCTCGA-3′ and Bim reverse 5′-TCAATGGAGTCTCCACACAC-3′. Primers for β-actin were the same as used for the generation of Northern blot probes (see above).

Western blot analysis and immunocytochemistry. Western blotting was done on primary CML cells and cells lines (K562, KU812, and Ton.B210-X cells) as described (29) using a polyclonal rabbit antibody against BimEL (Sigma; ref. 23), a polyclonal rabbit anti-Bad antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and an anti-β-actin antibody (Sigma). Immunocytochemistry was done on cytosin preparations of primary leukemia cells obtained from seven patients with CML and eight with normal bone marrow. Immunocytochemical staining was conducted as described (29) using a polyclonal goat anti-Bim antibody (dilution 1:100; Santa Cruz Biotechnology) and a biotinylated rabbit-anti-goat IgG (Biocarta, San Diego, CA). As chromogen, alkaline phosphatase complex (Biocarta) was used. Antibody reactivity was made visible by Neofuchsin (Nichirei, Tokyo, Japan).
Determination of cell viability and \(^3\)H-thymidine uptake. The percentage of apoptotic cells was quantified on Wright-Giemsa-stained cytosin preparations using conventional cytometric criteria (32). Cell viability was determined by trypan blue exclusion. For determination of \(^3\)H-thymidine uptake, cells were cultured in 96-well microtiter plates (5 × 10^4 cells per well) in the absence or presence of various concentrations of STI571 (60 nmol/L to 1 μmol/L), AMN107 (10 pmol/L to 1 μmol/L), or MG132 (1 μmol/L to 100 μmol/L) for 48 hours. To determine cooperative drug effects, various combinations of inhibitors were applied. After incubation, 1 μCi \(^3\)H-thymidine was added. Twelve hours later, cells were harvested on filter membranes, and bound radioactivity measured in a \(\beta\)-counter (Top-Count NXT, Packard Bioscience, Meriden, CT). All experiments were done in triplicates.

Statistical analysis. To determine the level of significance in cell growth, paired Student's t test was applied. Results were considered to be significantly different, when \(P < 0.05\). To determine synergistic effects of MGI32 and STI571, and of MG132 and AMN107, combination index values were calculated according to published guidelines using a commercially available software (Calcusyn; Biosoft, Ferguson, MO; ref. 33).

Results

Expression of Bim in normal bone marrow cells and patients with chronic myelogenous leukemia. As assessed by Western blotting, normal bone marrow cells were found to express the Bim protein in a constitutive manner (Fig. 1A). Exposure of these cells to IL-3 resulted in down-regulation of Bim expression (Fig. 1A). In patients with CML, bone marrow cells expressed significantly lower levels of Bim compared with normal bone marrow cells, which was demonstrable by Western blotting and densitometry (CML: 19 ± 19% of normal cells = 100%, \(P < 0.05\); Fig. 1B) as well as by immunocytochemistry (Fig. 1C). In addition, CML cells expressed lower levels of Bim mRNA compared with normal bone marrow cells by Northern blotting and densitometry (normal bone marrow, \(n = 4\), 100 ± 27%; CML, \(n = 4\), 23 ± 16%; \(P < 0.05\); see also Fig. 1D). Effects of imatinib and AMN107 on expression of Bim in chronic myelogenous leukemia cells. Exposure of primary CML cells to imatinib (STI571, 1 μmol/L) led to a substantial up-regulation in expression of the Bim protein (270 ± 70% of control by densitometry of Western blots; see also Fig. 2A). The same effect was seen with the novel BCR/ABL tyrosine kinase inhibitor AMN107 (34), applied at 100 nmol/L (Fig. 2A, right). Inhibition of BCR/ABL also led to a substantial increase in expression of bim mRNA in CML cells as determined by Northern blotting and densitometry (imatinib: 370 ± 240% compared with control medium = 100%, \(n = 3\)). A typical Northern blot experiment is shown in Fig. 2B. Corresponding data were obtained with two CML-derived cell lines (i.e., K562 and KU812). In particular, as assessed by densitometry of Western and Northern blots, imatinib increased the expression of the Bim protein (K562: 390 ± 200% of control = 100%, \(n = 3\); KU812: 210 ± 90% of control, \(n = 3\)) and expression of bim mRNA (K562: 430 ± 190% of control, \(n = 3\); KU812: 340 ± 140%, \(n = 3\)) in these cells. Almost the same effect was obtained with AMN107 (Fig. 2C-D). However, AMN107 was found to induce up-regulation of Bim in these cells at a lower concentration compared with imatinib as expected from recent studies (34).

BCR/ABL down-regulates expression of Bim in Ton.B210-X cells. To provide definitive evidence for the role of BCR/ABL in “Bim suppression,” Ton.B210-X cells were employed. In these experiments, doxycycline-induced expression of BCR/ABL was found to be associated with a substantial decrease in expression of the Bim protein (Fig. 3A) and a corresponding decrease in expression of bim mRNA (Fig. 3B). As visible in Fig. 3A, the BCR/ABL-induced decrease in expression of Bim in these cells was abrogated by addition of either imatinib (1 μmol/L) or AMN107 (100 nmol/L). In control experiments, doxycycline did not influence expression of Bim in parental (nontransfected) Ba/F3 cells (data not shown).

The BCR/ABL-induced decrease in expression of Bim was found to be due to a posttranscriptional mechanism. In fact, induction of BCR/ABL did not lead to a decrease in bim promoter activity in Ton.B210-X cells (bim promoter activity in doxycycline-exposed cells: 120 ± 14% compared with control, \(n = 3\)). In a next step, we applied the RNA synthesis inhibitor actinomycin D. This agent induced a time-dependent decrease in expression of bim mRNA in Ton.B210-X cells, which was much more rapid and pronounced in doxycycline-induced (BCR/ABL expressing) Ton.B210-X cells than in cells kept in control medium (Fig. 3C and D). These results suggest that the BCR/ABL-induced decrease in expression of Bim is a posttranscriptional event.

BCR/ABL-induced down-regulation of Bim in leukemic cells involves the Ras/Raf/MEK/ERK pathway. To characterize signal transduction pathways contributing to BCR/ABL-dependent down-regulation of Bim, pharmacologic inhibitors of MEK (PD98059), PI3K

![Figure 1.](image-url)
BCR/ABL Down-regulates Bim

Role of the proteasome in BCR/ABL-induced down-modulation of Bim. Recent data suggest that BCR/ABL down-regulates Bim in K562 cells through a degradation pathway involving the proteasome. The BCR/ABL effect on Bim-down-regulation via the proteasome in K562 cells can be augmented significantly by addition of PMA (23). In this study, we were able to confirm the involvement of the proteasome in BCR/ABL-induced down-regulation of Bim in Ton.B210-X cells. In fact, incubation of BCR/ABL-expressing Ton.B210-X cells with the proteasome inhibitor MG132 resulted in an increased expression of the Bim protein (Fig. 5). The same effect of MG132 was seen with K562 and KU812 cells (data not shown) confirming the data of Luciano et al. (23). In addition, we were able to show that MG132 up-regulates expression of Bim in primary CML cells (Fig. 5B). We then compared the effects of MG132 and imatinib on constitutive expression of Bim and on the PMA-induced decrease (by degradation through the proteasome) of Bim in K562 cells. In these experiments, we found that the PMA-induced degradation of Bim is effectively counteracted by MG132 (Fig. 5C) but is not counteracted in the same way by imatinib (Fig. 5D). We next asked whether MG132 would also up-regulate Bim expression in imatinib-resistant leukemic cells. In a first step, we analyzed Ba/F3 cells expressing various imatinib-resistant mutants of BCR/ABL. As shown in Fig. 6A, MG132 produced a substantial increase in expression of the Bim protein in all Ba/F3 subclones examined. In addition, we found that MG132 up-regulates Bim expression in primary leukemic cells obtained from a patient with imatinib-resistant CML in the same way as AMN107 (Fig. 6B). These data show that proteasome inhibition may be an effective approach to counteract Bim suppression in imatinib-resistant leukemic cells.

Effects of MG132 on growth of BCR/ABL-transformed cells. The striking effects of MG132 on Bim expression prompted us to examine the effects of this proteasome inhibitor on growth of CML cells. As shown in Fig. 7, MG132 inhibited the proliferation of K562 cells (IC50, 30–100 nmol/L, see Fig. 7A) and of KU812 cells (IC50, 30–100 nmol/L; see Fig. 7B) in a dose-dependent manner. Moreover, MG132 was found to counteract 3H-thymidine uptake in primary CML cells obtained from a patient with imatinib-resistant disease (Fig. 7C) as well as in Ba/F3 cells expressing various imatinib-resistant mutants of BCR/ABL without significant differences in IC50 values (ranging at about 100 nmol/L) among the mutants examined (Fig. 7D).

Finally, we asked whether MG132 and imatinib would produce synergistic effects on growth of imatinib-sensitive and/or imatinib-resistant cells. However, neither imatinib nor AMN107 were found to synergize with MG132 in producing growth inhibition in K562 cells, KU812 cells, or in Ba/F3 cells expressing imatinib-resistant mutants of BCR/ABL (data not shown).

Inhibition of drug-induced reexpression of Bim by small interfering RNA rescues leukemic cells from drug-induced apoptosis. To show functional significance of Bim reexpression induced by imatinib or MG132 in K562 cells, a bim-specific siRNA was applied. In control experiments, this siRNA produced an almost

Figure 2. Effects of imatinib (STI571) and AMN107 on expression of Bim in primary CML cells and CML-derived cell lines. A, Western blot analysis of expression of Bim in primary CML cells exposed to control medium versus imatinib/STI571 (1 μmol/L, left), or control medium versus AMN107 (100 nmol/L, right) for 24 hours. Western blotting was done using a polyclonal anti-Bim antibody. Equal loading is shown by probing for β-actin. B, Northern blot analysis of bim mRNA expression in primary CML cells after exposure to control medium or imatinib/STI571 (1 μmol/L) for 12 hours. Northern blot analysis was done using cDNA probes described in the text. The β-actin loading control is also shown. C, Western blot analysis of K562 cells exposed to control medium or AMN107 (100 nmol/L) for 24 hours. D, Western blot analysis of KU812 cells exposed to control medium or AMN107 (100 nmol/L) for 24 hours.
complete knockdown of Bim in HL60 cells but did not down-regulate expression of Bad (another BH3-only protein) by Western blotting (data not shown). After transfection of K562 cells with bim siRNA, both the imatinib-induced and the MG132-induced expression of Bim were significantly reduced in Western blot experiments when compared with expression of Bim in nontransfected K562 cells or K562 cells transfected with a control siRNA against luciferase (Fig. 8A-B). The siRNA-induced decrease in Bim expression was found to rescue K562 cells as well as primary CML cells from imatinib-induced apoptosis, although the effect of bim siRNA on growth inhibition in primary cells was less pronounced compared with K562 cells (Fig. 8C-D). Interestingly, the siRNA-induced decrease in Bim was also found to counteract MG132-induced apoptosis (Fig. 8C) as well as PD98059-induced apoptosis in K562 cells (not shown). These data suggest that "reexpressed" Bim plays a functional role as a drug-induced death regulator in CML cells.

Effect of enforced Bim expression in primary chronic myelogenous leukemia cells. To provide further evidence for

Figure 3. Effect of BCR/ABL on expression of Bim in Ton.B210-X cells. A, Ton.B210-X cells were grown in control medium, medium and doxycycline alone (BCR/ABL), doxycycline plus imatinib (1 μmol/L), or doxycycline plus AMN107 (100 nmol/L) for 24 hours. After incubation, cells were examined for expression of the Bim protein by Western blotting. Equal loading was confirmed by probing for β-actin. B, Northern blot analysis of bim mRNA expression in Ton.B210-X cells exposed to control medium or doxycycline (BCR/ABL). h-Actin served as loading control. C and D, time course of bim mRNA expression in Ton.B210-X cells. Ton.B210-X cells were grown in the presence of doxycycline (+ BCR/ABL; C) or in control medium (– BCR/ABL; D) for 16 hours. Cells were then split and incubated in the absence (Co) or in the presence of actinomycin D (10 μg/mL) for 1, 2 or 3 hours (h). Thereafter, total RNA was isolated and subjected to Northern blotting using a murine BimEL cDNA probe. The β-actin loading control is also shown. C and D, left, representative Northern blot experiments; right, the densitometric evaluation of bim mRNA expression levels (normalized to β-actin). Columns, means of three independent experiments; bars, ±SD.

Figure 4. Effects of signal transduction inhibitors on expression of Bim in Ton.B210-X cells and primary CML cells. A, Ton.B210-X cells were grown in control medium (Control) or in the presence of doxycycline (for induction of BCR/ABL). BCR/ABL" cells (+ Doxycycline) were cultured in control medium (BCR/ABL) or in the presence of PD98059 (50 μmol/L), LY294002 (20 μmol/L), rapamycin (20 nmol/L), or imatinib (STI571, 1 μmol/L) for 12 hours. Thereafter, RNA was isolated and subjected to Northern blotting. Expression of bim mRNA was detected using a murine BimEL cDNA probe. The β-actin loading control is also shown. B, Ton.B210-X cells were grown in control medium or in the presence of doxycycline (+ Doxycycline). Doxycycline-exposed (BCR/ABL") cells were incubated without (BCR/ABL) or with the aforementioned inhibitors (same type and dose as in A) for 24 hours. Thereafter, cells were isolated and subjected to Western blotting. Expression of the Bim protein was examined by using a Bim-specific antibody. The β-actin loading control is also shown (bottom). Western blot analysis (C) and Northern blot analysis (D) of expression of Bim in primary CML cells. Western blotting was done using a polyclonal anti-Bim antibody, and Northern blotting by using a bim-specific cDNA probe. Before being analyzed, cells were incubated in control medium, PD98059 (50 μmol/L), LY294002 (20 μmol/L), or rapamycin (20 nmol/L) for 12 hours (Northern blotting) or 24 hours (Western blotting) at 37 °C and 5% CO₂. Equal loading was confirmed by probing for β-actin.
cells transduced with pWPT-BimL stably expressed bim mRNA, for 24 hours. After incubation, cells were collected and examined for expression of the Bim protein by Western blotting. Equal loading was confirmed by probing for -actin. As visible in Fig. 8CML cells was associated with a time-dependent increase in the percentage of apoptotic cells (Fig. 8E).

As assessed by fluorescence microscopy, about 65% of all leukemic cells were GFP positive after infection. As visible in Fig. 8D, differential effects of MG132 and imatinib/STI571 on expression of Bim in K562 cells treated with PMA. K562 cells were first incubated in control medium or in medium containing either MG132 (25 μmol/L, C) or imatinib (STI571, 1 μmol/L; D) for 45 minutes. PMA (10 ng/mL) was added to the medium (alone or together with MG132 or imatinib) for various time periods as indicated. After incubation, cells were examined for expression of the Bim protein by Western blotting. Equal loading was confirmed by probing for -actin.

Abbreviation: P-Bim, phosphorylated Bim EL.

The role of Bim as a death regulator, primary CML cells were transduced with a BimL-cDNA by lentiviral-mediated gene transfer. As assessed by fluorescence microscopy, about 65% of all leukemic cells were GFP positive after infection. As visible in Fig. 8E, CML cells transduced with pWPT-BimL stably expressed bim mRNA, whereas CML cells transduced with pWPT-GFP (control) did not express bim mRNA. As expected, enforced expression of Bim in CML cells was associated with a time-dependent increase in the percentage of apoptotic cells (Fig. 8E).

Discussion

Abnormal expression of the death activator Bim has recently been implicated in survival of leukemic cell lines (19, 20, 22–24). In the current report, we show that primary leukemic cells isolated from patients with CML express lower levels of Bim compared with normal bone marrow cells, and that the CML-specific oncprotein BCR/ABL down-regulates expression of Bim in Ba/F3 cells. Our data also show that various pharmacologic agents, including BCR/ABL tyrosine kinase inhibitors and the proteasome inhibitor MG132, lead to reexpression of Bim in leukemic cells and thereby may produce growth inhibition. Thus, reexpression of Bim may represent a novel attractive strategy to counteract antiapoptotic mechanisms in CML cells. This concept is further supported by the demonstration that enforced expression of Bim in primary CML cells by lentiviral-mediated gene transfer is associated with induction of apoptosis.

Although Bim expression has already been analyzed in BCR/ABL+ cell lines (22–24), it was of pivotal importance to determine the levels of Bim in primary CML cells. The results of our study show that primary CML cells express significantly lower levels of bim mRNA and Bim protein compared with normal bone marrow cells. Interestingly, low level expression of Bim was seen in chronic-phase CML as well as in the accelerated phase of the disease. In addition, we observed low-level expression of Bim in the BCR/ABL+ cell lines K562 and KU812. However, Bim expression may not only be regulated by oncogenic molecules but also by physiologic stimuli. Likewise, IL-3 was found to down-regulate expression of Bim in normal bone marrow cells, confirming previous data (22). The multiple regulators of Bim expression may also explain the variability in expression of Bim found in normal bone marrow cells and CML cells in this study.

Previous studies have already shown that the BCR/ABL kinase inhibitor imatinib promotes expression of Bim in K562 cells (23, 24). This Bim-promoting effect of imatinib in K562 cells was confirmed in the present study. In addition, we were able to show that imatinib promotes expression of Bim in KU812 cells and primary CML cells.

The effect of MG132 on expression of Bim in imatinib-resistant cells. A, Ba/F3 cells stably expressing wild-type BCR/ABL (Ba/F3p210wt) or imatinib-resistant BCR/ABL mutants were incubated in control medium (Control), MG132 (1 μmol/L), or STI571 (1 μmol/L) at 37°C and 5% CO2 for 12 hours. After incubation, cells were examined for expression of the Bim protein by Western blotting. Equal loading was confirmed by probing for -actin. B, primary leukemic cells obtained from a patient with imatinib-resistant CML (accelerated phase) were incubated with control medium, MG132 (25 μmol/L), or AMN107 (100 nmol/L) for 24 hours. Then, cells were examined for expression of the Bim protein by Western blotting. -Actin served as a loading control.
and the same effect was observed with the novel BCR/ABL tyrosine kinase inhibitor AMN107 (34). Interestingly, this new BCR/ABL-targeting drug was found to induce reexpression of Bim at lower concentrations compared with imatinib, which is in line with the superior growth-inhibitory effect of this agent (34).

To further show that BCR/ABL down-regulates expression of Bim in leukemic cells, we employed Ba/F3 cells with doxycycline-inducible expression of BCR/ABL (Ton.B210-X). In these cells, expression of BCR/ABL was found to be associated with down-regulation of Bim, an effect that was reverted by imatinib and AMN107. Together with our data on primary CML cells, these results formally establish that BCR/ABL directly suppresses the expression of Bim in leukemic cells.

A number of different signal transduction pathways are involved in BCR/ABL-dependent growth and survival of CML cells (4–8). In the present study, we asked which of these pathways would contribute to BCR/ABL-induced down-regulation of Bim. Our experiments show that the MEK inhibitor PD98059 counteracts the BCR/ABL-induced down-regulation of Bim in Ton.B210-X cells, whereas the PI3K and mTOR inhibitors applied showed little if any effects. In addition, PD98059 was found to promote expression of Bim in primary CML cells as well as expression of Bim in K562 and KU812 cells. All in all, these data suggest that the BCR/ABL-induced suppression of Bim in leukemic cells involves the MAPK pathway, confirming previous data (23, 24).

Recent data suggest that depending on the type of cell and status of stimulation, Bim expression is regulated by transcriptional as well as posttranscriptional/posttranslational mechanisms (19–24, 35–38). In this study, we asked whether the BCR/ABL-induced down-regulation of Bim in Ton.B210-X cells is regulated by a...
transcriptional or a posttranscriptional mechanism. Our results show that BCR/ABL does not regulate Bim promoter activity in Ba/F3 cells. In addition, the actinomycin D–induced decrease in Bim mRNA expression was more pronounced and faster in BCR/ABL-expressing Ba/F3 cells compared with Ba/F3 cells lacking BCR/ABL. These data suggest that BCR/ABL down-regulates Bim in leukemic cells through a posttranscriptional mechanism and probably through modulation of mRNA stability.

A number of recent studies have shown that Bim levels are regulated not only through modulation of mRNA stability but also by proteasomal degradation (23, 38–41). Such degradation may particularly involve the BimEL splice variant, which is phosphorylated in response to physiologic stimuli or oncogenes such as BCR/ABL, before its proteasomal degradation (23). In the present study, we were able to show that inhibition of the proteasome (by MG132) is associated with a substantial increase in expression of Bim in primary CML cells, in K562 and KU812 cells, and in Ba/F3 cells expressing BCR/ABL. The effect of MG132 on “Bim reexpression” in K562 cells was similar in magnitude compared with STI571 and AMN107 and was also seen after pretreatment of cells with PMA. This is of particular interest, because imatinib was unable to induce reexpression of Bim in PMA-stimulated K562 cells. This observation was a first hint that proteasome inhibitors may induce reexpression of Bim in CML cells that have lost their responsivity to imatinib.

Because resistance to imatinib is an emerging problem in the treatment of CML (42–46), the above observation also prompted us to examine the effects of the proteasome inhibitor MG132 on growth of imatinib-resistant CML cells. In these experiments, we were able to show that MG132 inhibits the growth of primary CML cells in patients with imatinib-resistant disease. In addition, MG132 was found to down-regulate the growth of Ba/F3 cells expressing various imatinib-resistant mutants of BCR/ABL, without major differences in IC50 values when comparing drug-resistant cells with imatinib-sensitive cells. These effects of MG132 were also found to parallel Bim reexpression and may suggest a new treatment option in CML, which has also been proposed by other investigations using inhibitors of the proteasome (47, 48). Another interesting aspect in this regard is that Bim suppression by a specific siRNA not only inhibited the imatinib-induced reexpression of Bim in K562 cells which is consistent with the data of Kuribara et al. (24) but also counteracted the growth-inhibitory effects of the proteasome inhibitor MG132 in these cells. These data suggest that Bim plays an important role as death regulator in drug-exposed cells. However, on the other hand, down-modulation of Bim by the siRNA applied did not completely inhibit the imatinib-induced or the MG132-induced apoptosis in K562 cells. This observation suggests that apart from Bim other death regulators may be involved in drug-induced apoptosis in CML cells.

In a next step, we asked whether MG132 and imatinib or MG132 and AMN107, would exert synergistic inhibitory effects on leukemic cell growth. Unexpectedly, however, no synergistic effects were found. The reason for this result remains unknown. The most
apparent explanation would be that BCR/ABL-dependent phosphorylation and the consecutive proteosomal degradation of Bim represents the only major pathway and mechanism underlying Bim suppression in leukemic cells (23). If so, any cooperative drug effects would be expected to be additive in nature but not synergistic. An alternative explanation for the non synergistic effects of these drugs would be that cellular proteins countering responses of leukemic cells to imatinib are also degraded via the proteasome pathway. Up-regulation of Bim expression by a proteasome inhibitor may be particularly useful in patients presenting with the T315I mutation of BCR/ABL, which is resistant to all kinase inhibitors currently used in clinical trials (34).

In summary, our data show that primary CML cells express lower levels of Bim compared with normal bone marrow cells, and that the BCR/ABL oncoprotein down-regulates expression of Bim through the MEK-signaling pathway and posttranslational mechanisms involving the proteasome. Forced expression of Bim by novel signal transduction inhibitors or by proteasome-targeting agents may represent an attractive approach to counteract growth of neoplastic cells in patients with imatinib-resistant CML.

Acknowledgments

Received: 3/22/2005; revised: 8/2/2005; accepted: 8/8/2005.

Grant support: Fonds zur Förderung der Wissenschaftlichen Forschung, Austria; NIH (grant GM46259); and Cancer Research UK (grant C3013/A28913).

We thank Yves Guérin and Kingsley A. Ojo for reviewing the manuscript.

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Karl J. Aichberger, Matthias Mayerhofer, Maria-Theresa Krauth, et al.


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