A BCR/ABL-INDEPENDENT, LYN-DEPENDENT FORM OF IMATINIB MESYLATE (STI-571) RESISTANCE IS ASSOCIATED WITH ALTERED EXPRESSION OF BCL-2

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SUMMARY

The relationship between the Src kinase Lyn and Bcl-2 expression was examined in chronic myelogenous leukemia cells (K562 and LAMA84) displaying a Bcr/Abl-independent form of imatinib mesylate resistance. K562-R and LAMA-R cells that were markedly resistant to induction of mitochondrial dysfunction (e.g., loss of mitochondrial membrane potential, Bax translocation, cytochrome c and AIF release) and apoptosis by imatinib mesylate exhibited a pronounced reduction in expression of Bcr/Abl, Bcl-xL, and STAT5, but a striking increase in levels of activated Lyn. Whereas basal expression of Bcl-2 protein was very low in parental cells, imatinib-resistant cells displayed a marked increase in Bcl-2 mRNA and/or protein levels. Treatment of LAMA-R cells with the Src kinase inhibitor PP2 significantly reduced Lyn activation as well as Bcl-2 mRNA and protein levels. Transient or stable transfection of LAMA84 or K562 cells with a constitutively active Lyn (Y508F), but not with a kinase-dead mutant (K275D), significantly increased Bcl-2 protein expression and protected cells from lethality of imatinib mesylate. Ectopic expression of Bcl-2 protected K562 and LAMA84 cells from imatinib mesylate- and PP2-mediated lethality. Conversely, interference with Bcl-2 function by co-administration of the small molecule Bcl-2 inhibitor HA14-1, or down-regulation of Bcl-2 expression by siRNA or antisense strategies significantly increased mitochondrial dysfunction and apoptosis induced by imatinib mesylate and the topoisomerase inhibitor VP-16 in LAMA-R cells. In marked contrast, these interventions had little effect in parental LAMA84 cells which display low basal levels of Bcl-2. Together, these findings indicate that activation of Lyn in leukemia cells displaying a
Bcr/Abl-independent form of imatinib mesylate resistance plays a functional role in Bcl-2 up-regulation, and provide a theoretical basis for the development of therapeutic strategies targeting Bcl-2 in such a setting.
INTRODUCTION

Chronic myelogenous leukemia (CML) is a hematopoietic stem cell disorder in which 90% of patients display a reciprocal translocation involving chromosomes 9 and 22, resulting in what has been designated the Philadelphia (Ph) chromosome. This translocation stems from a head-to-tail fusion of the breakpoint cluster region (bcr) at band q34 of chromosome 22 with the proto-oncogene (c-abl) at band q34 of chromosome 9. This leads in turn to the expression in virtually all Ph+ CML patients of the chimeric fusion protein p210 Bcr/Abl, a constitutively active tyrosine kinase (1). The etiologic role of the Bcr/Abl oncogenic tyrosine kinase in the pathogenesis of CML has been well documented (2,3). Expression of Bcr/Abl not only contributes to leukemic transformation but also represents a barrier to the successful treatment of this disorder. For example, Bcr/Abl stimulates diverse downstream survival signal transduction pathways, including those related to STAT5 (signal transducer and activator of transcription 5), Ras/Raf/MEK (mitogen-activated protein kinase kinase)/ERK (extracellular signal regulating kinase) and PI3K (phosphatidylinositol 3-kinase)/Akt that, collectively, provide affected cells with survival and proliferative advantages (4,5). Secondly, Bcr/Abl confers resistance to DNA-damage triggered by diverse chemotherapeutic drugs and radiation (6,7). The anti-apoptotic effects of Bcr/Abl can stem from increased expression of pro-survival proteins e.g., STAT5-mediated induction of Bcl-xL and A1 (8,9) or post-translational modifications, including the PI-3K/Akt-mediated inactivating phosphorylation of Bad (10).
Imatinib mesylate (STI-571, Gleevec, CGP57148B) is a 2-phenylamino pyrimidine that targets the ATP-binding site of the kinase domain of Abl (11). Imatinib mesylate is lethal to Bcr/Abl-positive cells in culture (12,13); moreover, this agent has proven highly effective in patients with chronic phase CML, and, to a lesser extent, in patients with accelerated or blast phase disease (14,15). Resistance to imatinib mesylate stems from bcr/abl gene amplification, leading to overexpression of Bcr/Abl protein (16,17), or point mutations in the bcr/abl gene, resulting in a single amino acid substitution (e.g., 315 Thr – Ile or 255 Glu – Lys or Val) within the ATP pocket of the Abl kinase domain known to be essential for imatinib mesylate binding (17,18). In addition, mutations outside of the kinase domain that allosterically inhibit imatinib mesylate binding to the Bcr/Abl protein have now been described (19).

Recently, a putatively Bcr/Abl-independent form of imatinib mesylate resistance has been reported by several groups (20-22). Specifically, Bcr/Abl-positive CML cells cultured in the continuous presence of imatinib mesylate (20,21), or obtained from certain CML patients who have progressed while receiving imatinib mesylate (22), display a decline in Bcr/Abl protein and/or mRNA levels and a corresponding increase in expression/activity of the Lyn and Hck kinase (21,22). Lyn and Hck represent members of the Src tyrosine kinase family, and have been implicated in the regulation of cell survival and proliferation, among other numerous other functions (23,24). Lyn exists in two isoforms, p56 and p53, derived from alternatively spliced mRNAs (25). The catalytic activity of Lyn is tightly regulated through tyrosine phosphorylation at the conserved Try508 (26). Evidence derived from both murine and human model systems suggest that Lyn is a potentially important downstream target of Bcr/Abl (27); moreover, Lyn has
been shown to be activated in Bcr/Abl-positive cells obtained from CML patients in blast crisis as well as in HL-60 human leukemia cells transfected with Bcr/Abl (28). Together, such findings raise the possibility that activation of Lyn can subserve the anti-apoptotic functions of Bcr/Abl, including conditions in which expression of Bcr/Abl, is, for whatever reason, diminished.

Although the role that Lyn plays in protecting cells from lethal stimuli has been examined in some detail (29), the functional relationship that exists between this Src kinase and Bcr/Abl remains to be fully elucidated. Moreover, no information exists concerning interactions hyperactivated Lyn might have with Bcl-2 family members. To address these issues, we have examined the molecular profile of human CML cells that have become resistant to imatinib mesylate in association with loss of Bcr/Abl and activation of the Lyn kinase. Here we report that in such cells, activation of Lyn is associated with a pronounced increase in levels of the anti-apoptotic protein Bcl-2, and that disruption of Lyn activation through either pharmacologic or genetic strategies has a significant functional impact on Bcl-2 expression and resistance to mitochondria-dependent apoptosis. In addition, pharmacological or genetic disruption of Bcl-2 function increases the susceptibility of these resistant cells to lethality of imatinib mesylate or the topoisomerase II inhibitor VP-16. These findings may have implications for the development of new therapeutic strategies directed against leukemia cells exhibiting novel forms of imatinib mesylate resistance.
MATERIALS AND METHODS

Cells and Reagents - LAMA84 were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Imatinib-resistant LAMA84 cells (LAMA-R) were generated by culturing cells in gradually increasing concentrations of imatinib mesylate (beginning at 0.1 μM and increasing in stepwise increments of 0.1 μM) until a level of 1 μM (30). Parental K562 and imatinib-resistant K562-R cells (generated as described above for LAMA-R cells) were kindly provided by Dr. L Varticovski (Tufts University School of Medicine, Boston, MA). Cells were cultured in 10% FBS/RPMI 1640 medium as described previously (30). K562-R and LAMA-R were maintained in the medium containing 1μM imatinib mesylate, and washed free of drug before all experimental procedures. All experiments were performed utilizing logarithmically growing cells (4-6 x 10^5 cells/ml).

Imatinib mesylate (Gleevec, STI-571) was provided by Dr. Elizabeth Buchdunger, Novartis Pharmaceuticals, Basel, Switzerland, dissolved in DMSO at a stock concentration of 50mM and stored at –20°C, and subsequently diluted with serum-free RPMI medium prior to use. PP2 (4-amino-5-[4-chlorophenyl]-7-[t-butyl]pyrazolo[3,4-d]pyrimidine), a selective inhibitor of Src family of tyrosine kinases (31), PP3 (4-amino-7-phenylpyrazolo[3,4-d]pyrimidine), a negative control for PP2, HA14-1 (ethyl-2-amino-6-bromo-4-[1-cyano-2-ethoxy-2-oxoethyl]-4H-chromene-3-carboxylate), a cell permeable and low molecular weight Bcl-2 inhibitory ligand (32,33), were purchased from Calbiochem (San Diego, CA) and Biomol (Plymouth Meeting, PA), respectively. cpm-1285 (a cell-permeable Bcl-2 binding peptide derived from the BH3
domain encompassing residues 140-165) and its negative control peptide cpm-1285mt (a mutant in which Leu 151 is replaced by Ala) (34) were provided by Calbiochem. Etoposide (VP-16), a topoisomerase II inhibitor, was obtained from Sigma (St. Louis, MO). These agents were dissolved in DMSO as a stock solution, stored at –80°C. In all experiments, the final concentration of DMSO did not exceed 0.1%.

**Transient and Stable Transfection with cDNAs** – cDNAs encoding full-length, kinase-defective (K275D, in which Asp is substituted for Lys 275 in the ATP-binding pocket), and constitutively active (Y508F, in which Tyr 508 is substituted in the conserved tail of the C-terminus with Phe) Lyn were subcloned into pcDNA3 containing an HA-tag (26). Bcl-2 cDNA (wild type) in pUSEamp was purchased from Upstate Biotechnology (Lake Placid, NY). LAMA84 and K562 cells (1x10⁶ per condition) were transiently transfected with three forms of Lyn cDNA (i.e., wild-type, K275D and Y508F), and Bcl-2 cDNA, respectively, using the Amaxa Nucleofector Device (program T-16) with Cell Line Specific Nucleofector Kit V (Amaxa GmbH, Cologne, Germany). Alternatively, K562 and LAMA84 cells were transfected with K275D and Y508F constructs as well as their empty vector counterparts (pcDNA3), respectively, and stably transfected clones were selected by limited dilution using G418.

**Bcl-2 RNA Interference and Antisense Oligonucleotides** – 1x10⁶ LAMA84 and LAMA-R cells were transfected with 2 µg Bcl-2 annealed dsRNAi oligonucleotide (5’-GUACAUCCAUUAAGCGUGTT-3’/3’-TTCAUGUAGGUAUAUUCGAC-5’), Orbigen, San Diego, CA) and SignalSilence Control siRNA (Cell Signaling, Beverly, MA), respectively, using the Amaza Nucleofector Device (program T-16, Kit V). Alternatively, LAMA84 and LAMA-R cells were transfected with 5 µg of a Bcl-2
antisense oligonucleotides (5’-TCTCCCAGCGTGCGCCAT-3’, G3139, Calbiochem) or a scrambled control oligonucleotide (5’-TACCGCGTGCGACCCTCT-3’, G3622, Calbiochem) as described for transfection with dsRNAi. Transfection efficiency (> 80% at 24 hr post-transfection) was monitored by using fluorescein-labeled dsRNAi or antisense oligonucleotide and flow cytometric analysis. Bcl-2 protein levels were determined by Western blot analysis.

**Apoptosis and Viability** - The extent of apoptosis was evaluated by Annexin V-FITC staining and flow cytometry. Briefly, 1x10^6 cells were stained with Annexin V-FITC (BD PharMingen, San Diego, CA) and 5µg/ml propidium iodide (PI, Sigma, St. Louis, MO) in 1x binding buffer (10mM Hepes/NaOH, pH7.4, 140mM NaOH, 2.5mM CaCl_2) for 15 min at room temperature in the dark. The samples were analyzed by flow cytometry within 1 hr to determine the percentage of cells displaying Annexin V^+ (early apoptosis) or Annexin V^+/PI^+ staining (late apoptosis). The ViaCount assay was performed to evaluate cell viability in the experiments involving transient transfection. 1x10^5 cells were stained with Guava ViaCount Reagent, and the percentage of viable cells determined using a Guava Personal Cytometer (Guava Technologies, Hayward, CA) as per the manufacturer’s instructions. The ViaCount assay distinguishes between viable and non-viable cells based on the differential permeability of DNA-binding dyes in the ViaCount Reagent, and was found to correlate closely with the results of Annexin/PI staining.

**Mitochondrial Membrane Potential (ΔΨm)** - 2x10^5 cells were stained with 40nM 3,3-dihexyloxacarbocyanine (DiOC_6; Molecular Probes Inc., Eugene, OR) in PBS at 37°C for 20 min, and then analyzed by flow cytometry. The percentage of cells exhibiting
decreased level of DiOC₆ up-take, which reflects loss of ΔΨₘ, was determined using Becton-Dickinson FACScan (Becton-Dickinson, San Jose, CA).

**Cell Growth and Survival (MTT Assay)** - 5x10⁴ (in 100µl volume)/well cells were seeded into 96-well plate and incubated with 20µl of CellTiter 96® AQueous One Solution (Promega, Madison, WI) as per the manufacturer’s instructions, and the absorbance at 490nm recorded using a 96-well plate reader (Molecular Devices, Sunnyvale, CA).

**Western Blot** - Whole-cell pellets were lysed in SDS sample buffer and 30µg protein for each condition was subjected to Western blot analysis following the procedures previously described in detail (35). Where indicated, the blots were reprobed with antibodies against β-actin (rabbit polyclonal, Transduction Lab., Lexington, KY) or α-tubulin (mouse monoclonal, Calbiochem) to ensure equal loading and transfer of proteins. The following antibodies were used as primary antibodies: phospho-Bcr (Tyr177) antibody (rabbit polyclonal, Cell Signaling), c-Abl antibody (mouse monoclonal, Santa Cruz, Santa Cruz, CA), STAT5 antibody (rabbit polyclonal, Santa Cruz), Bcl-xL antibody (rabbit polyclonal, Cell Signaling), anti-PARP (poly [ADP-ribose] polymerase, mouse monoclonal, Biomol), phospho-Lyn (Tyr507) antibody (rabbit polyclonal, Cell Signaling), Lyn antibody (rabbit polyclonal, Cell Signaling), anti-human Bcl-2 oncoprotein (mouse monoclonal, Dako, Carpinteria, CA), Bax antibody (rabbit polyclonal, Santa Cruz), XIAP antibody (mouse monoclonal, Transduction Lab.), phospho-CrkL (Tyr207) antibody (rabbit polyclonal, Cell Signaling), CrkL (32H4)
antibody (mouse monoclonal, Cell Signaling), phospho-Hck (Tyr411) antibody (rabbit polyclonal, Santa Cruz), Anti-Hck antibody (rabbit polyclonal, Upstate Biotechnology, Lake Placid, NY), HA-probe (rabbit polyclonal, Santa Cruz). In some cases, the density of blots was quantified using FluoChem 8800 Imaging System (Alpha Innotech, San Leandro, CA) and VideoTesT-Master software (VideoTesT, Ltd., St.-Petersburg, Russia).

Translocation of Bax, Cytochrome c and AIF - 4 x 10^6 cells were washed in PBS and lysed by incubating for 30 seconds in lysis buffer (75 mM NaCl, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM EDTA, and 350 µg/ml digitonin). After centrifuged at 12,000g for 1 min, the supernatant (cytosolic fraction) was collected in an equal volume of 2x sample buffer, and the pellet (mitochondria-rich fraction) were lysed by sonication in 1x SDS sample buffer. For both cytosolic and pellet fractions, the proteins were quantified, and 30µg protein per condition was separated by 15% SDS-PAGE and subjected to Western blot as described above, using Bax antibody, cytochrome c antibody (mouse monoclonal, Santa Cruz) and AIF (apoptosis-inducing factor) antibody (mouse monoclonal, Santa Cruz) as primary antibody.

RT-PCR - Total RNA was isolated from 1x10^6 cells using RNeasy Mini Kit (Qiagen, Valencia, CA) with QIAshredder spin column (Qiagen) as per the manufacturer’s instructions. 1µg per condition of total RNA was subjected to RT-PCR reaction using OneStep RT-PCR Kit (Qiagen) and PTC-200 Peltier Thermal cycler (MJ Research, Reno, NV). The primers (forward: 5’-CGACTTCGCGAGATGTCAGCCAGCAG-3’; reverse: 5’-
GACCCACGGATAGACCCGGTGTTCA-3’) were used for Bcl-2. RT-PCR was performed under the conditions as follows: reverse transcription at 50°C for 30 min, initial PCR activation step at 95°C for 15 min, three-step cycling (denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min) for 30 cycles, and final extension at 72°C for 10 min. The reactions were, in parallel, run for actin as endogenous control. PCR products of Bcl-2 (388bp) were analyzed in 2 % agarose gel with ethidium bromide.

Real-Time Quantitative RT-PCR - Total RNA was prepared as described above. The real-time RT-PCR was performed on the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using the TaqMan One Step PCR Master Mix Reagents Kit (P/N: 4309169). All the samples were tested in triplicate under the conditions recommended by the fabricant. The cycling conditions were: 48°C/30 min; 95°C/10 min; and 40 cycles of 95°C/15 sec and 60°C/1 min. The cycle threshold was determined to provide the optimal standard curve values (0.98 to 1.0). the probes (5’-CCTGGTGGACAACATCGCCCTGT-3’) and primers for Bcl-2 (forward: 5’-CCTGGTGGACAACATCGCCCTGT-3’; reverse: 5’-GCCGGTTTCAGGTACTCAGTCAT-3’) were designed using the Primer Express 2.0 version. The probes were labeled at the 5’ end with FAM (6-carboxyfluoresceine) and at the 3’ end with TAMRA (6-carboxytetramethylrhodamine). Ribosomal RNA (18S rRNA) from the Pre-developed TaqMan Assay Reagents (P/N: 4310893E) was used as endogenous control.
Statistical Analysis - For analysis of apoptosis, ΔΨm, MTT, viability, and real-time quantitative RT-PCR, values represent the means ± SD for at least three separate experiments performed in triplicate. The significance of differences between experimental variables was determined using the Student’s t test.
RESULTS

*Imatinib-resistant CML cells exhibit loss of Bcr/Abl and down-regulation of Bcr/Abl-downstream targets* - The response of K562 and LAMA84 cells and their resistant counterparts (K562-R and LAMA-R) to imatinib mesylate was compared. Following an exposure to 0.5 - 10.0 µM imatinib, K562 (48 hr) and LAMA84 cells (24 hr) displayed progressive increases in Annexin V/PI-positivity, whereas their resistant counterparts were minimally affected (Figures 1A and 1B). Thus, both the K562-R and LAMA-R cell lines displayed marked resistance to imatinib mesylate-mediated lethality.

Attempts were then made to establish the basis for imatinib mesylate resistance in the two resistant cell lines. Parental K562 and LAMA84 cells, as well as their resistant counterparts, were exposed to 1 µM imatinib mesylate for 48 hr (K562 and K562-R) and 24 hr (LAMA84 and LAMA-R), after which Western blot analysis was performed. As shown in Figure 1C, both K562-R and LAMA-R cells displayed a dramatic reduction in levels of total and phosphorylated Bcr/Abl, as well as markedly diminished expression of Stat5 and Bcl-xL, well-described Bcr/Abl downstream targets (36), compared to parental cells. Treatment of both sensitive and resistant cells with imatinib mesylate resulted in reductions in Bcl-xL levels, whereas expression of the inhibitor of apoptosis protein XIAP was only diminished following imatinib mesylate exposure in sensitive cells. Consistent with the results shown in Figures 1A and 1B, imatinib mesylate-induced PARP cleavage was largely abrogated in K562-R and LAMA-R cells. Thus, both K562-R and LAMA-R cells appeared to exhibit a Bcr/Abl-independent form of imatinib mesylate...
resistance, analogous to that previously described by Donato et al., in K562 cells and patient samples (21,22).

Bcr/Abl-independent imatinib-resistance is associated with marked activation of Lyn and increased expression of Bcl-2 - Expression of phosphorylated Bcr/Abl and related proteins in resistant cells was examined next. As shown in Figure 2A, levels of phospho-Bcr/Abl (activated) were dramatically reduced in K562-R and LAMA-R compared to their parental counterparts. Furthermore, consistent with earlier results involving K562 cells (21), phosphorylation of the Src kinase Lyn was markedly increased in both resistant cell lines. Levels of total Lyn were also increased in both resistant cell lines. However, in striking contrast to the reduction in Bcl-xL expression exhibited by K562-R and LAMA-R cells (Figure 1C), Bcl-2 expression, basal levels of which were very low in parental K562 and LAMA84 cells, was dramatically increased in the resistant cell lines (Figure 2A). In separate studies, no marked changes in the expression of other apoptotic regulatory proteins (e.g. Mcl-1, survivin, XIAP) could be detected in resistant cells (data not shown). Thus, acquisition of the Bcr/Abl-independent, Lyn-activated phenotype was associated with down-regulation of Bcl-xL but a pronounced increase in the basal levels of Bcl-2 expression.

To gain insights into the mechanism by which Bcl-2 levels were increased in LAMA-R cells exhibiting increased Lyn activation, RT-PCR and real-time RT-PCR were employed. As shown in Figure 2B and as quantified in Figure 2C, levels of Bcl-2 mRNA were 4-fold greater in the resistant cell line than in parental controls (P < 0.001). Similar results were obtained in K562-R cells (data not shown).
Imatinib mesylate does not diminish Lyn activation or Bcl-2 expression, and fails to induce mitochondrial dysfunction in LAMA-R cells - The impact of imatinib mesylate on the expression/activation of Lyn and on the disposition of mitochondrial apoptotic regulatory proteins was examined next. As shown in Figure 3A, exposure to imatinib mesylate did not modify the marked increase in levels of total and phospho-Lyn characteristic of LAMA-R cells. LAMA-R cells also displayed an increase in levels of the phosphorylated form of the Src kinase Hck compared to parental cells. However, in contrast to the case of Lyn, expression of phospho-Hck was diminished in both sensitive and resistant cells following imatinib mesylate exposure. In addition, imatinib mesylate also reduced expression of the phosphorylated form of the docking protein CrkL in imatinib mesylate-sensitive and -resistant cells.

Treatment of LAMA84 or LAMA-R cells with imatinib mesylate did not affect total expression of Bcl-2 or Bax in either cell line. Treatment of parental LAMA84 cells with imatinib mesylate did, however, result in a marked redistribution of Bax from the cytosol to the pellet (mitochondrial fraction), whereas no changes were observed in LAMA-R cells (Figure 3B). An increase in Bcl-2 expression in the pellet was noted in LAMA-R cells, concordant with the increase in total Bcl-2 shown in Figure 2A, but this did not change with imatinib mesylate exposure. Consistent with the previous results, imatinib mesylate treatment resulted in a clear redistribution of cytochrome c and AIF from the pellet to the cytosol in LAMA84 cells, but not in LAMA-R cells. Western blot analysis of whole cell pellets revealed that imatinib mesylate did not modify total levels of Bcl-2, Bax, AIF, or cytochrome c in either cell line. Collectively, these findings are
compatible with the notion that the marked increase in mitochondrial-associated Bcl-2 in LAMA-R cells may contribute to protection against imatinib mesylate-mediated mitochondrial dysfunction. This concept was supported by results of studies monitoring changes in mitochondrial membrane potential, which revealed that LAMA-R cells were essentially immune to imatinib mesylate-mediated loss of ΔΨm compared to wild-type cells (Figure 3C).

**Inhibition of Lyn promotes apoptosis and down-regulates Bcl-2 in LAMA-R cells**

To investigate the functional relationship between enhanced Lyn activation and increased Bcl-2 expression, the Src kinase inhibitor PP2 was employed (31). Consistent with earlier results involving imatinib-resistant K562 cells displaying diminished Bcr/Abl expression (21), imatinib-resistant, Bcr/Abl-independent LAMA-R cells were more susceptible to apoptosis induced by 10 µM PP2 than their parental counterparts (Figure 4A; 68.0% vs 35.5%; P < 0.05). In contrast, exposure to the inactive derivative PP3 did not trigger cell death in either sensitive or resistant cells. As shown by the Western blot analysis in Figure 4B, PP2 markedly reduced levels of phospho-Bcr/Abl in wild-type LAMA84 cells and phospho-Lyn in Bcr/Abl-independent LAMA-R cells. PP2 modestly reduced total Bcr/Abl expression but had no effect on levels of total Lyn. Significantly, exposure of LAMA-R cells to 10 µM PP2 reduced Bcl-2 mRNA levels by > 60% (P < 0.001 compared to untreated cells), accompanied by a reduction in Bcl-2 protein expression (Figure 4C). Together, these findings suggest that activation of the Lyn kinase in Bcr/Abl-independent, imatinib-resistant LAMA84 cells plays a functional role in enhanced Bcl-2 expression.
Transfection with wild-type or constitutively active Lyn up-regulates Bcl-2 expression in CML cells - To confirm the functional relationship between Lyn activation and Bcl-2 up-regulation, transient and stable transfection strategies were employed. First, LAMA84 cells were transiently transfected with wild-type Lyn cDNA and kinase-defective (K275D) or constitutively active (Y508F) Lyn mutants, and Bcl-2 expression monitored by Western analysis (Figure 4D). Transient transfection of cells with the wild-type, the constitutively active, or the kinase-defective mutant resulted in a clear increase in Lyn expression as well as expression of the HA tag. However, whereas cells transfected with the constitutively active mutant or wild-type construct exhibited a pronounced increase in Bcl-2 expression, no changes were observed in cells transfected with the kinase-defective construct. Thus, enforced activation of Lyn in Bcr/Abl+ leukemia cells was associated with a clear increase in Bcl-2 expression.

Parallel studies were carried out in K562 and LAMA84 cells stably transfected with the constitutively active (Y508F) or kinase dead (K257D) Lyn constructs. Both transfected cells lines exhibited a marked increase in Lyn expression (Figure 5A). On the other hand, expression of the Bcr/Abl protein was similar in parental and transfected cell lines. However, whereas Y508F-transfected K562 and LAMA84 cells displayed a pronounced increase in Bcl-2 protein levels, K275D-transfected cells did not. In contrast to alterations in Bcl-2 expression, no changes in levels of Bcl-xL were noted. Furthermore, stable transfection with constitutively active Lyn (Y508F) significantly protected K562 and LAMA84 cells from mitochondrial dysfunction (loss of ∆Ψm; data not shown) and apoptosis (Figure 5B and 5C) induced by 0.5 µM imatinib mesylate (P <
0.02 in each cell line), while the kinase-dead mutant did not. Together, these findings argue strongly that Lyn activation plays a significant functional role in enhanced Bcl-2 expression, and raise the possibility that these events contribute to imatinib mesylate resistance.

_Bcl-2 inhibition sensitizes Lyn-activated CML cells to imatinib mesylate_ - To investigate the functional role that induction of Bcl-2 might play in regulating the imatinib mesylate response of Lyn-activated LAMA84 and K562 cells displaying diminished expression of Bcr/Abl, HA14-1, a small molecule Bcl-2 inhibitor (32) was used. This cell permeable non-peptidic ligand of the hydrophobic Bcl-2 surface pocket interferes with the ability of Bcl-2 to block the actions of pro-apoptotic BH3 domain-only family members (e.g., Bak), and in so doing, promotes apoptosis either by itself or in combination with cytotoxic agents (33,37,38). As shown in Figures 5B and 5C, co-administration of HA14-1 (10 µM), which by itself was minimally toxic, failed to potentiate imatinib mesylate-mediated loss of ∆Ψm (data not shown) or apoptosis in K562 and LAMA84 cells transfected with empty vector and kinase-defective Lyn (K275D), but strikingly increased imatinib mesylate-mediated lethality in constitutively active Lyn (Y508F)-transfected cells displaying increased expression of Bcl-2. Such findings provide indirect evidence that increased Bcl-2 expression in Lyn-activated cells may contribute to imatinib mesylate resistance.

_Imatinib mesylate down-regulates phospho-Bcr/Abl, Bcl-xL, and XIAP expression in Lyn-activated K562 cells, but does not modify levels of phospho-Lyn and Bcl-2_
Comparisons were then made between the effects of imatinib mesylate on the expression of various signaling and apoptotic regulatory proteins in K562 cells stably transfected with either constitutively active (Y508F) or inactive (K275D) Lyn (Figure 5D). Whereas imatinib mesylate diminished expression of phospho-Bcr/Abl in all cell lines, in none of the cell lines it did modify expression of phospho-Lyn. Furthermore, imatinib mesylate failed to alter the pronounced increase in Bcl-2 expression in Lyn-activated cells (Y508F). In contrast, imatinib mesylate induced clear reductions in expression of Bcl-xL and XIAP in each of the cell lines. Finally, imatinib mesylate-mediated PARP degradation was attenuated in Y508F-transfected cells compared to cells transfected with both empty vector and the K275D mutant. The failure of imatinib mesylate to diminish increased expression of Bcl-2 in Lyn-activated cells is consistent with a role for this protein in the development of imatinib mesylate resistance.

Enforced expression of Bcl-2 protects CML cells from imatinib mesylate- and PP2-mediated lethality - The preceding studies provided indirect evidence that alterations in expression of Bcl-2 might contribute to imatinib mesylate resistance in Lyn-activated cells. To test this hypothesis more directly, LAMA84 and K562 cells were transiently transfected with cDNA constructs encoding the wild type Bcl-2 protein, and resulting effects on the response of cells to imatinib mesylate and the Src kinase inhibitor PP2 were monitored. As shown by the Western blots in Figure 6A (left panels), K562 and LAMA84 cells transfected with the Bcl-2 cDNA displayed marked increases in Bcl-2 protein levels, but no changes in expression of phospho-Lyn. Furthermore, Bcl-2 transfectants of both LAMA84 and K562 were significantly more resistant to the lethal
effects of imatinib mesylate (1 µM) than their control counterparts, P < 0.05 and 0.01 (Figure 6A, right panel), respectively. LAMA84 and K562 cells ectopically expressing Bcl-2 were also significantly less sensitive to the lethal effects of PP2, which was previously shown to down-regulate Bcl-2 expression in wild-type cells (Figure 4C), compared to their control counterparts (Figure 6A; right panel). These findings provide support for the notion that increased Bcl-2 expression in Lyn-activated K562 and LAMA84 cells contributes functionally to imatinib mesylate resistance.

Down-regulation of Bcl-2 expression by siRNA or antisense oligonucleotides sensitizes LAMA-R but not parental LAMA84 cells to imatinib mesylate lethality - To gain further insights into the functional significance of increased Bcl-2 expression in imatinib resistance in Lyn-activated cells, siRNA and antisense strategies were employed. Transient transfection of LAMA-R cells with either Bcl-2 siRNA or Bcl-2 antisense oligonucleotides resulted in substantial declines in Bcl-2 protein expression (Figure 6B and 6C, upper panels). In contrast, basal expression of Bcl-2 was barely discernible in parental cells. Consistent with the latter findings, transfection with Bcl-2 siRNA or Bcl-2 antisense oligonucleotide did not have a significant effect on the survival of parental LAMA84 cells following exposure to imatinib mesylate. In marked contrast, LAMA-R cells, which displayed a pronounced reduction in Bcl-2 expression following transfection with Bcl-2 siRNA or a Bcl-2 antisense oligonucleotide, exhibited a dramatic increase in imatinib mesylate lethality, P < 0.01 compared to untreated controls (Figures 6B and 6C, lower panels). Concordant results were obtained in K562-R cells (data not shown). It is also noteworthy that down-regulation of Bcl-2 expression by either siRNA or antisense
oligonucleotides significantly reduced the viability of LAMA-R cells (P < 0.01 compared to control siRNA-transfected cells), but had no effect on parental LAMA cells (Figure 6B and 6C, lower panels). Similar results were obtained in K562-R and their parental K562 counterparts (data not shown). These findings suggest that the survival of imatinib mesylate-resistant cells exhibiting diminished Bcr/Abl expression but increased Lyn activation and Bcl-2 expression may depend critically on Bcl-2 function. Taken in conjunction with the preceding observations, these findings also argue that increased Bcl-2 expression in Lyn-activated CML cells exhibiting reduced Bcr/Abl expression plays a significant functional role in imatinib mesylate resistance.

The small molecule Bcl-2 inhibitor HA14-1 is more lethal to LAMA-R than to parental cells and sensitizes LAMA-R cells to imatinib mesylate-mediated lethality - The preceding findings (Figure 5B and 5C) demonstrated that treatment with the small molecule Bcl-2 inhibitor HA14-1 sensitized parental K562 or LAMA84 cells transiently transfected with cDNA encoding constitutively active (but not kinase-defective) Lyn to imatinib mesylate lethality. Attempts were then undertaken to determine whether a similar phenomenon occurred in imatinib mesylate-resistant CML cells displaying increased Lyn activation in conjunction with diminished Bcr/Abl expression (Figure 2A). As shown by the dose-response curves in Figure 7A, LAMA-R and K562-R cells were significantly more sensitive than their parental counterparts to the growth inhibitory effects of various concentrations of HA14-1, as determined by the MTT assay. LAMA-R and K562-R cells were also significantly more susceptible to HA14-1-mediated apoptosis than parental controls (Figure 7B).
Consistent with previous results shown in Figure 1, Annexin V/PI analysis revealed that 0.5 or 1 µM imatinib mesylate (24 hr) were essentially non-toxic to LAMA-R cells (data not shown). However, while 10 µM HA14-1 by itself only modestly induced cell death (19% of cells), combined treatment with HA14-1 and imatinib mesylate resulted in a dramatic increase in lethality (to > 50% of cells; Figure 7C). Corresponding Western blot analysis revealed that while 0.5 or 1 µM imatinib mesylate (data not shown) and 10 µM HA14-1 administered individually failed to induce PARP cleavage in LAMA-R cells, combined treatment resulted in pronounced PARP degradation (Figure 7D). No significant changes in Lyn phosphorylation were noted with any treatment. Together, these findings indicate that as in the case of cells transfected with a constitutively active Lyn construct, co-administration of subtoxic concentration of HA14-1 strikingly increases imatinib mesylate lethality in LAMA-R cells displaying increased Lyn activation and Bcl-2 expression.

Co-administration of imatinib mesylate and HA14-1 in LAMA-R cells results in the pronounced release of cytochrome c and AIF into the cytosol accompanied by mitochondrial translocation of Bax - The effects of HA14-1 on imatinib mesylate-mediated mitochondrial events in LAMA-R cells were examined next. Consistent with the previous results shown in Figure 3B, exposure of LAMA-R cells to 0.5 or 1 µM imatinib mesylate alone had no effect on the intracellular disposition of Bax, which resided primarily in the cytosol, or on cytochrome c and AIF, which were primarily localized to the mitochondrial fraction (data not shown). Similarly, 10 µM HA14-1 alone exerted very modest effects on Bax translocation or cytochrome c/AIF redistribution.
(Figure 7E). However, when the agents were combined, Bax underwent a pronounced redistribution from the cytoplasm to the mitochondria, a phenomenon characteristically associated with apoptosis (39). This event was accompanied by the reciprocal translocation of cytochrome c and AIF from the mitochondria to the cytosol. Combined treatment was also associated with the faint appearance of a Bcl-2 cleavage product, particularly in the mitochondrial fractions (Figure 7E). These findings suggest that disruption of Bcl-2 function by HA14-1 in LAMA-R cells restores, at least in part, the capacity of imatinib mesylate to induce mitochondrial dysfunction and redistribution of pro-apoptotic Bcl-2 family members in otherwise imatinib mesylate-resistant cells.

*Inhibition or down-regulation of Bcl-2 increases the sensitivity of LAMA-R cells to the topoisomerase II inhibitor VP-16* - To determine whether increased expression of Bcl-2 in Lyn-activated cells might also play a role in determining the sensitivity of imatinib mesylate-resistant cells to other cytotoxic agents, cells were exposed to the topoisomerase inhibitor VP-16 following transient transfection with Bcl-2 siRNA or Bcl-2 antisense oligonucleotides as described for results displayed in Figures 6B and 6C. As shown in Figure 8A, the loss of cell viability induced by VP-16 (5 μM, 24 hr) was significantly greater in LAMA-R cells transfected with Bcl-2 siRNA or Bcl-2 antisense oligonucleotides compared to those transfected with control vectors (P < 0.01 in each case). Similar results were obtained in K562-R cells (data not shown). Parallel studies involving the small molecule Bcl-2 inhibitor HA14-1 were performed in parental LAMA84 and LAMA-R cells (Figure 8B). Co-administration of HA14-1 (10 μM) with varying concentrations of VP-16 resulted in a dramatic increase in apoptosis in LAMA-R
cells, but exerted minimal effects in parental LAMA84 controls, which express very low basal levels of Bcl-2 (Figure 2A). Interestingly, LAMA-R cells were somewhat more susceptible to the lethal actions of VP-16 alone compared to parental cells, presumably reflecting loss of the protective effects of Bcr/Abl and/or Bcl-xL (40). Parallel results were obtained when loss of ∆Ψm was monitored (data not shown).

Consistent with these results, co-administration of HA14-1 (10 µM) with VP-16 (5 µM) in LAMA-R cells resulted in a marked redistribution of Bax from the cytosol to the mitochondrial fractions, accompanied by the pronounced release of cytochrome c and AIF into the cytosol, whereas minimal changes were observed in cells exposed to the agents individually (Figure 8C). These events were not observed in parental cells (data not shown). No changes in total cellular levels of Bax, Bcl-2, AIF, or cytochrome c were noted for all conditions. Finally, co-administration of a Bcl-2 binding peptide (cpm-1285), which consists of a fatty acid chemically attached to a peptide derived from the pro-apoptotic protein Bad, and which has previously been shown to disrupt Bcl-2 function (34), substantially increased VP-16-mediated mitochondrial dysfunction (loss of ∆Ψm) in LAMA-R cells, but exerted no effect in the parental line (Figure 8D). A control peptide (cpm-1285mt) was without effect. Taken together with the preceding results, these findings suggest that increased expression of Bcl-2 in Bcr/Abl-diminished, Lyn-activated CML cells plays a significant functional role in protecting cells from mitochondrial dysfunction and apoptosis resulting from various environmental stresses, including exposure to cytotoxic agents such as imatinib mesylate and VP-16.
DISCUSSION

Despite the success of imatinib mesylate in Bcr/Abl-positive malignancies, particularly CML (14,15), the development of resistance to this agent remains a continuing problem. Imatinib mesylate resistance can take several forms, including mutations in the Bcr/Abl kinase domain resulting in interference with imatinib mesylate binding (17,18), and bcr/abl amplification (16,17) and/or increased Bcr/Abl protein expression (16,20). In addition, mutations outside of the Bcr/Abl kinase domain have also been shown to confer imatinib mesylate resistance (19). Recently, however, several groups have described resistant cell lines and importantly, patient specimens displaying diminished Bcr/Abl expression (20-22), accompanied by increased activation of the Lyn kinase (21). Loss of Bcr/Abl in this form of imatinib-resistance appears to occur at the transcriptional level (20). Although activation of Lyn (21) and up-regulation of Bcl-2 (20) have been individually observed in Bcr/Abl-independent cells displaying imatinib mesylate resistance, a functional link between these events has not previously been described. In this context, Sinha et al. observed increased Bcl-2 protein levels accompanying Lyn activation in murine myeloid cells (32Dcl3) transfected with v-Cbl (41). The present results argue that in imatinib-resistant K562 cells displaying diminished expression of Bcr/Abl, activation of Lyn plays a significant role in Bcl-2 up-regulation, and suggest that strategies targeting Bcl-2 in cells displaying Lyn activation warrants further investigation.

Although a relationship between Lyn and Bcr/Abl in the pathogenesis of CML is supported by several lines of evidence (27,28), genetic and biochemical studies suggest
that Lyn may exert either pro- or anti-apoptotic functions, depending on cell context. For example, genotoxic stimuli (e.g., ionizing radiation and ara-C) activate the Lyn kinase, leading to induction of cell death through the Lyn-dependent activation of SAPK/JNK (26,42). On the other hand, Lyn has been shown to play a key role in the transduction of antiapoptotic signals initiated by growth factors and cytokines, at least in vitro (43,44). In support of this model, Lyn negatively regulates the proapoptotic function of GADD34 in HEK293 and Hela cells undergoing apoptosis in response to DNA damage (29).

Furthermore, Lyn and Fgr protein kinases block retinoic acid-induced apoptosis and promote granulocytic differentiation in HL-60 cells (45). In a bcr/abl-transfected murine myeloid cell line, p53/p56lyn displayed a marked increase in activity compared to parental cells; moreover, p53/p56lyn kinase activity is significantly higher in Bcr/Abl-positive myeloid cell lines (e.g., K562, BV173 and LAMA84) than in Bcr/Abl-negative myeloid cell lines such as JOSK-M (27). Furthermore, phosphorylation of Bcr/Abl was associated with Lyn activation in bcr/abl-transfected HL-60 cells (28). Finally, in CML cells, Lyn may mediate pathological crosstalk between Bcr/Abl and CXCR4, a G protein-coupled receptor of the stromal-derived factor pathway, thereby disrupting chemokine signaling and chemotaxis (28). Collectively, these findings suggest that in hematopoietic cells, Lyn represents an important downstream target of Bcr/Abl, and raise the possibility Lyn may subsume some of the anti-apoptotic functions of this oncoprotein.

The results described here indicate, for the first time, that Lyn activation plays a functional role in Bcl-2 up-regulation exhibited by cells displaying Bcr/Abl-independent forms of imatinib mesylate resistance. In the study by Nimminaiapalli et al., loss of Bcr/Abl in K562 cells was associated with down-regulation of STAT5 as well as with
reduced expression of Bcl-xL (20), consistent with our results in K562-R and LAMA-R cells. In contrast to the down-regulation of these proteins, K562 exhibiting diminished expression of Bcr/Abl displayed increased expression of Bcl-2 (20). However, the activation status of Lyn was not specifically examined in this study. In the report by Donato et al., Lyn activation in imatinib mesylate-resistant Bcr/Abl-independent K562 cells as well as patient-derived cells was also associated with down-regulation of STAT5, but Bcl-2 expression was not examined (21). In the present study, several lines of evidence argue strongly for a causal relationship between Lyn activation and up-regulation of Bcl-2. First, LAMA-R cells exhibiting a pronounced activation of Lyn displayed a dramatic increase in levels of Bcl-2 mRNA and protein. Second, transient transfection of wild-type LAMA84 cells or stable transfection of wild-type K562 and LAMA84 cells with a constitutively active (but not a kinase-defective) Lyn construct resulted in an increase in Bcl-2 protein. Third, exposure of LAMA-R cells to the Src kinase inhibitor PP2 down-regulated Bcl-2 mRNA and protein expression. Collectively, these findings are consistent with the notion that Lyn activation plays a significant functional role in Bcl-2 up-regulation in these cells. In this context, evidence of increased Bcl-2 promoter activity or diminished ubiquitination of Bcl-2 was not observed in v-Cbl-transfected 32Del3 exhibiting enhanced basal Lyn activation and increased Bcl-2 expression compared to control cells (38). The mechanism by which Lyn activation increases Bcl-2 expression in K562-R and LAMA-R cells displaying reduced Bcr/Abl expression is presently unknown, and to the best of our knowledge, this association has not previously been reported. However, results of the RT-PCR studies suggest that Lyn-mediated up-regulation of Bcl-2 may occur at the transcriptional level. The possibility
exists that activation of Lyn, a Src family tyrosine kinase, may act directly or indirectly (for example, through downstream signaling pathways) to activate certain transcription factors (e.g., NF-κB, Myb, C/EBP, CREB) known to be major transcriptional regulators of Bcl-2 (46-49). Attempts to explore the mechanistic link between Lyn activation and Bcl-2 expression are currently underway.

It is noteworthy that Bcl-2 has been shown to cooperate with Bcr/Abl to promote leukemogenesis, and to result in a more aggressive tumor phenotype (50,51). Although increased Bcl-2 expression has been observed in imatinib-resistant K562 cells displaying loss of Bcr/Abl (20), the functional role of this phenomenon has not been investigated. The present results suggest that increased Bcl-2 expression in Lyn-activated leukemia cells helps to protect them from various environmental stresses, particularly under conditions in which the cytoprotective functions of Bcr/Abl are lost. Significantly, HA14-1, a small molecule Bcl-2 inhibitor, was primarily effective in reducing cell viability in the LAMA-R cell line, indicating that Bcl-2 plays an important role in cell survival in this setting. In contrast, HA14-1 had relatively little impact on the survival of parental Bcr/Abl-positive cells, which express very low to absent levels of Bcl-2. One possibility is that increased expression of Bcl-2 in, Lyn-activated cells displaying reduced expression of Bcr/Abl compensates for the down-regulation or inactivation of survival factors (e.g., Bcl-xL, STAT5, Akt, and MAPK) that accompanies loss of Bcr/Abl (20,21).

Several lines of evidence suggest that increased Bcl-2 expression in Lyn-activated, Bcr/Abl-diminished CML cells contributes to the imatinib mesylate-resistant phenotype. First, the ability of HA14-1 to promote imatinib mesylate lethality in LAMA-R cells suggests, albeit indirectly, a functional role for Bcl-2 in drug resistance. Second,
down-regulation of Bcl-2 expression by either RNA interference or antisense strategies restored the ability of imatinib mesylate to trigger cell death in otherwise resistant cells. Third, transient transfection of K562 and LAMA cells with cDNA encoding Bcl-2 significantly protected them from imatinib mesylate-mediated lethality. Such findings are consistent with recent reports suggesting that strategies that interfere with Bcl-2 function (e.g., antisense approaches) may enhance the lethal effects of imatinib mesylate in Bcr/Abl+ leukemia cells (52). The mechanism by which imatinib mesylate exerts its lethal effects toward leukemia cells exhibiting diminished Bcr/Abl expression remains to be determined, although it may be relevant that in such cells, residual Bcr/Abl kinase activity has been detected (21). An alternative possibility is that increased Bcl-2 expression may protect against the lethal consequences of inhibition of other kinases by imatinib mesylate (53). Whatever the mechanism, the present findings suggest that strategies targeting Bcl-2 may be effective in overcoming certain forms of imatinib mesylate resistance associated with Lyn activation and Bcl-2 up-regulation.

It is also noteworthy that co-administration of minimally toxic concentrations of HA14-1 dramatically enhanced the lethality of an agent other than imatinib mesylate (e.g., the topoisomerase inhibitor VP-16), to trigger mitochondrial dysfunction and apoptosis in LAMA-R cells. Similar results were noted with Bcl-2 siRNA and antisense strategies. Specifically, HA14-1 promoted VP-16-mediated translocation of Bax to the mitochondria and release of cytochrome c and AIF into the cytoplasm of LAMA-R cells, whereas VP-16 administered alone was largely ineffective. Furthermore, comparable results were obtained when a Bcl-2-binding peptide (cpm-1285) was employed. This peptide was designed by chemically attaching a fatty acid to a peptide derived from the
proapoptotic protein Bad, allowing the compound to enter cells and bind Bcl-2 (34). Although the mechanism by which Bcl-2 blocks apoptosis remains the subject of debate, interference with the ability of BH3 domain-only molecules (e.g., Bid, BAD, BIM, and NOXA) to activate multidomain proapoptotic members (e.g., BAX and BAK), thereby preventing the release of pro-apoptotic proteins (e.g., cytochrome c, Smac/Diablo, and AIF), represents a leading candidate (54). It has been reported that HA14-1 binds to the Bcl-2 BH3 domain, thereby disrupting interactions with pro-apoptotic proteins such as Bax and Bak (32). Together, these findings support the concept that up-regulation of Bcl-2 in Bcr/Abl-down-regulated, Lyn-activated leukemia cells may act in a more general manner to promote cell survival in the face of environmental stresses, including DNA-damaging agents.

The observation that Bcr/Abl-independent, Lyn-activated forms of imatinib mesylate resistance are associated with up-regulation of Bcl-2 has potential implications for the rational design of targeted strategies in certain forms of drug-resistant CML. Clinical experience to date suggests that imatinib mesylate resistance is most frequently related to Bcr/Abl mutations (e.g., 70%), and less often to gene amplification and/or increased protein expression (55). While sporadic cases have been reported (22), the frequency with which Bcr/Abl-independent, Lyn-activated forms of imatinib mesylate resistance occurs in patients is not known at this time. In any case, resistant cells expressing mutant Bcr/Abl protein may retain their sensitivity to kinase inhibitors (e.g., PD166326, PD180970, and CGP76030) other than imatinib mesylate (21,56,57). Alternatively, resistant cells in which Bcr/Abl expression is increased may be vulnerable to a strategy combining imatinib mesylate with other novel agents such as histone
deacetylase inhibitors (e.g. SAHA (58) or LAQ824 (59)). On the other hand, a rational strategy directed against, Lyn-activated cells displaying reduced expression of Bcr/Abl might require specific inhibitors of Lyn (21), or alternatively, agents capable of circumventing Bcl-2 resistance. These include CDK inhibitors (e.g., Flavopiridol, which has been reported to act independently of Bcl-2 (60), as well as Bcl-2 antisense oligonucleotides, which diminish Bcl-2 levels (52,61). In particular, approaches capable of disrupting Bcl-2 function or reducing Bcl-2 expression may be effective in potentiating the response of such resistant cells to both imatinib mesylate as well as more conventional antileukemic agents (e.g., VP-16). Accordingly, attempts to explore the latter strategies in CML cells displaying Bcr/Abl-independent forms of imatinib mesylate resistance are currently underway.

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Figure 1. Imatinib-resistant cells exhibit loss of Bcr/Abl and down-regulation of STAT5 and Bcl-xL. K562 and LAMA84 cells were cultured in increasing concentrations of imatinib mesylate (starting at 0.1 μM and increased stepwise in increments of 0.1 μM) until concentrations of 1 μM were achieved. Resistant cells were isolated as described in Methods and designated as K562-R and LAMA-R. Imatinib-resistant cells were maintained in 10% FBS/RPMI 1640 medium containing 1 μM imatinib mesylate. Before
experiments, imatinib-resistant cells were washed free of drug. K562-R (A), LAMA-R (B) and their parental counterparts (K562 and LAMA84) were incubated with 0.5 - 10 μM imatinib mesylate (designated here and in all subsequent figures as STI for STI571) for 48 hr (K562) and 24 hr (LAMA84), respectively, after which Annexin V-FITC/PI staining and flow cytometry were performed to monitor apoptosis. Results represent the means ± S.D. for three separate experiments performed in triplicate. K562-R (C, left panels, 48 hr), LAMA-R (C, right panels, 24 hr), and their parental cells were treated with 1μM imatinib mesylate, after which whole cell lysates were subjected to Western blot analysis using phospho-Bcr/Abl, total Bcr/Abl, STAT5, Bcl-xL, XIAP and PARP antibodies. CF = cleavage fragment. For each condition, lanes were loaded with 30μg of protein. Blots were subsequently reprobed with anti-actin for normalization. Two additional studies yielded equivalent results.
Figure 2. Bcl-2 is markedly up-regulated in Bcr/Abl-independent imatinib-resistant cells in association with pronounced activation of Lyn. (A) Whole cell lysates

Figure 2
obtained from K562-R (left panels), LAMA-R (right panels) and their parental counterparts were subjected to Western blot analysis to monitor levels of phospho-Bcr/Abl, total and phospho-Lyn, and Bcl-2. For each condition, lanes were loaded with 30µg of protein. (B) Total RNA was extracted from LAMA84 and LAMA-R cells and subjected to RT-PCR analysis to monitor Bcl-2 mRNA levels as described in Methods. Actin mRNA was amplified to normalize data. A 100 bp DNA ladder (Invitrogen) was used as a marker (M). For A and B, results are representative for three separate experiments. (C) Bcl-2 mRNA from LAMA84 and LAMA-R cells was quantified by real-time RT-PCR analysis as described in Methods, and 18S rRNA was used as an endogenous control. Total RNA samples from U937 cells were used in parallel reactions as a standard control (value for Bcl-2 mRNA = 20.0). Results represent the means ± S.D. for three separate experiments performed in triplicate.
Figure 3. Imatinib mesylate triggers mitochondrial dysfunction in parental LAMA84 cells but not in LAMA-R cells. (A) LAMA84 and LAMA-R cells were incubated with 1 μM imatinib mesylate for 24 hr, after which Western blot analysis was
performed to monitor expression of total and phosphorylated Lyn, Hck and CrkL. For each condition, lanes were loaded with 30µg of protein. Blots were subsequently reprobed with anti-actin to ensure equivalent loading and transfer. (B) Cells were treated as described in panel A and lysed in digitonin buffer. Cytosolic (cytosol) and mitochondria-rich (pellet) fractions were then separated by centrifugation and subjected to Western blot analysis to assess distribution of Bax, Bcl-2, AIF and cytochrome c (right panels). In parallel, whole-cell lysates were subjected to Western blot to monitor total levels of these proteins. For each condition, lanes were loaded with 30µg of protein. For A and B, two additional experiments yielded equivalent results. (C) Alternatively, DiOC₆ staining and flow cytometry were performed to determine the percentage of cells exhibiting low dye uptake, reflecting loss of mitochondrial membrane potential (ΔΨₘ), in LAMA84 and LAMA-R cells following treatment as described in panel A. Results represent the means ± S.D. for three separate experiments performed in triplicate.
Figure 4

A

Ctrl      PP2      PP3
LAMA      7.2%  4.4%  7.2%  5.9%  7.4%  5.9%  8.0%  4.7%
LAMA-R    22.1% 13.4% 22.1% 13.4% 41.6% 26.4% 41.6% 26.4%
Annexin V-FITC

B

PP2       -  +  -  +
Bcr/abl   
 p-Bcr/abl 
 p-Lyn    
 Lyn       
 PARP      
 CF        
 Actin     

C

LAMA-R
PP2       -  +
Actin     
 Bcl-2     
 Bcl-2     
 18S       
 Bcl-2     
 18S       
 Real-time RT-PCR

D

LAMA (Transient)
Vehicle   wt-Lyn  Y508F  K275D
HA-tag    
 Lyn       
 Bcl-2     
 Bcl-2     
 Bcl-xL    
 Actin     

Annexin V-FITC

Figure 4
Figure 4. The Src kinase inhibitor PP2 inactivates Lyn and down-regulates Bcl-2 expression in LAMA-R cells, while transient transfection with wild-type or constitutively active Lyn leads to increased expression of Bcl-2. (A) LAMA84 and LAMA-R cells were incubated of the specific Src-family kinase inhibitor PP2 or its negative control PP3 (10 µM each) for 24 hr, after which apoptosis was evaluated by Annexin V/PI staining and flow cytometry. Values reflect the percentage of cells in the Annexin+ (early apoptotic; lower right) and Annexin+/PI+ (late apoptotic; upper right) quadrants. (B) Following treatment as described above, whole cell lysates were subjected to Western blot analysis to monitor the effects of PP2 on total and phosphorylated Bcr/Abl and Lyn, and cleavage of PARP. CF = cleavage fragment. For each condition, lanes were loaded with 30µg of protein. Blots were subsequently reprobed with anti-actin to ensure equivalent loading and transfer. (C) Total RNA and whole cell lysates were prepared and subjected to RT-PCR, real-time RT-PCR, and Western blot analysis as described in Figure 2, respectively, to monitor Bcl-2 mRNA and protein levels in LAMA-R cells following a 24-hr incubation with 10 µM PP2. For Western blot, 30µg of protein were loaded in each lane. Blots were subsequently reprobed with anti-actin for normalization. Density of Bcl-2 and actin protein bands was quantified using an imaging system as described in Methods. Values reflect the ratio of integrated densitometric determinations between untreated and PP2-treated LAMA-R cells. (D) LAMA84 cells were transiently transfected with wild-type Lyn, constitutively active (Y508F) and kinase-inactive (K275D) mutants, and an empty vector (pcDNA3), respectively, as described in Methods. After 48 hr, cells were lysed and subjected to Western blot analysis to monitor expression of HA-tag, Lyn, Bcl-2 and Bcl-xL. In each lane, 30µg of
protein was loaded, and blots were subsequently reprobed with anti–actin for normalization. For A-D, results are representative for three separate experiments.
Figure 5. Stable transfection of LAMA84 and K562 cells with constitutively active Lyn results in increased expression of Bcl-2 and imatinib mesylate resistance, which is attenuated by the small molecule Bcl-2 inhibitor HA14-1. (A) K562 (right panels).
and LAMA84 (left panels) cells were transfected with constitutively active (Y508F) or kinase-inactive (K275D) Lyn, or an empty vector (pcDNA3), after which clones were selected by limited dilution in the presence of G418. Western blot analysis was performed to monitor expression of phospho-Bcr/Abl, Bcl-2 and Bcl-xL in the selected clones exhibiting increased Lyn expression. (B-C) Stably transfected K562 (B) and LAMA84 (C) cells isolated from the selected clones described in panel A were exposed to 0.5 μM imatinib mesylate and 10 μM HA14-1 alone or in combination for 48 hr (K562) or 24 hr (LAMA84), after which Annexin V/PI staining and flow cytometry were performed to assess apoptosis. Results represent the means ± S.D. for three separate experiments performed in triplicate. # = significantly lower than values for empty vector-transfected cells exposed to 0.5 μM imatinib mesylate (# P < 0.02 and ## P < 0.01); * = significantly greater than values for Y508F-transfected cells exposed to imatinib mesylate in the absence of HA14-1 (* P < 0.02 and ** P < 0.01). (D) K562 cells stably transfected with an empty vector, Y508F/Lyn, or K275D/Lyn were incubated with 0.5 μM imatinib mesylate for 48 hr, after which Western blot analysis was performed to monitor expression of phosphorylated Lyn and Bcr/Abl, Bcl-2, Bcl-xL, XIAP, and PARP. CF = cleavage fragment. For A and D, each lane was loaded with 30μg of protein. Blots were subsequently reprobed with anti-tubulin to ensure equivalent loading and transfer. Results are representative of three separate experiments.
Figure 6. Ectopic expression of Bcl-2 confers resistance to imatinib mesylate and PP2 in wild type cells, while down-regulation of Bcl-2 sensitizes LAMA-R but not
LAMA84 cells to imatinib mesylate lethality. (A) LAMA84 and K562 cells were transiently transfected with Bcl-2 cDNA and an empty vector (pUSE), respectively. After 24 hr, Western blot analysis was performed to monitor expression of Bcl-2 (left panels). Alternatively, after a 6-hr recovery following transfection, transfected cells were exposed to 1 µM imatinib mesylate, 10 µM PP2 or its negative control PP3 for 24 hr (LAMA84) or 48 hr (K562), after which cell viability (right panel) was determined by using ViaCount staining and a Guava Personal Cytometer as described in Methods. Results represent the means ± S.D. for three separate experiments performed in triplicate. * = significantly greater than values for empty vector-transfected cells exposed to imatinib mesylate or PP2 (* P < 0.05; ** P < 0.01). (B-C) LAMA84 and LAMA-R cells were transfected with Bcl-2 dsRNAi and a control siRNA (B), or Bcl-2 antisense oligonucleotide and its control oligonucleotide (C) as described in Methods. After 24 hr, cells were lysed and subjected to Western Blot analysis to monitor levels of Bcl-2 (upper panels). Alternatively, the transfected cells, after 6-hr recovery, were incubated in either the absence or presence of 1 µM imatinib mesylate for 24 hr, after which cell viability (lower panels) was determined as described in panel A. Results represent the means ± S.D. for three separate experiments performed in triplicate. ## = significantly lower than values for control siRNA- (B) or control antisense oligonucleotide-transfected (C) cells (P < 0.01). ** = significantly lower than values for the same transfected cells in the absence of imatinib mesylate (P < 0.01). For Western blot analyses in A-C, the phosphorylation status of Lyn was also evaluated. Each lane was loaded with 30µg of protein. Blots were subsequently reprobed with anti-tubulin to ensure equivalent loading and transfer. Results are representative of three separate experiments.
Figure 7. HA14-1 is more lethal to imatinib mesylate-resistant K562-R and LAMA-R cells than their sensitive parental counterparts and increases imatinib mesylate-mediated mitochondrial damage and apoptosis in LAMA-R cells. (A-B) LAMA84 and K562 cells, and their imatinib mesylate-resistant counterparts were exposed to
indicated concentrations of HA14-1 for 24 hr, after which an MTT assay (A) and Annexin V/PI staining/flow cytometry (B) were performed to monitor cell growth/survival and apoptosis, respectively. Results represent the means ± S.D. for three separate experiments performed in triplicate. * (LAMA-R) and # (K562-R) = significantly lower than values for parental cells (* and # P < 0.05; ** and ### P < 0.01). (C) LAMA-R cells were incubated with 0.5 and 1 μM imatinib mesylate in the presence of 10 μM HA14-1 for 24 hr, after which apoptosis was evaluated by Annexin V/PI staining and flow cytometry. Values reflect the percentage of cells in the Annexin+ (early apoptosis; lower right) and Annexin+/PI+ (late apoptosis; upper right) quadrants. Results are representative of three separate experiments. (D-E) LAMA-R cells were treated as described in panel C, after which Western blot analysis was performed to assess expression of total and phosphorylated Lyn, PARP cleavage (D), and distribution of Bcl-2, Bax, AIF and cytochrome c in cytosolic (cytosol) and mitochondria-rich (pellet) fractions (E). CF = cleavage fragment. Each lane was loaded with 30μg of protein. As indicated, blots were subsequently reprobed with anti-actin to ensure equivalent loading and transfer. Two additional experiments yielded equivalent results.
Figure 8. Inhibition of Bcl-2 results in increased sensitivity to topoisomerase inhibitor etoposide in LAMA-R cells. (A) LAMA-R cells were transfected with Bcl-2 siRNA and antisense oligonucleotides as described in Figure 6B and 6C. After recovery for 6 hr, the transfected cells were exposed to 5 µM VP-16 for 24 hr, after which cell
viability was determined as described in Figure 6. Results represent the means ± S.D. for three separate experiments performed in triplicate. ** = significantly lower than values for control siRNA- or antisense oligonucleotide-transfected cells exposed to VP-16 (P < 0.01). (B) LAMA84 and their imatinib mesylate-resistant counterparts (LAMA-R) were exposed to the indicated concentrations of VP-16 in the presence or absence of 10 µM HA 14-1 for 24 hr, after which the percentage of apoptotic cells was assessed by Annexin V/PI staining and flow cytometry. Results represent the means ± S.D. for three separate experiments performed in triplicate. (C) LAMA-R cells were treated with 10 µM HA14-1 ± 5 µM VP-16 for 24 hr, after which whole cell lysates as well as cytosolic (cytosol) and mitochondria-rich (pellet) fractions were prepared and subjected to Western blot analysis to monitor distribution of Bax, Bcl-2, cytochrome c and AIF. For each condition, lanes were loaded with 30µg of protein. Results are representative of three separate experiments. (D) LAMA84 and LAMA-R cells were co-treated with 5 µM VP-16 and 50 µM of either the Bcl-2-binding peptide (cpm-1285) or a negative control peptide (cpm-1285mt) for 24 hr, after which DiOC₆ staining and flow cytometry were performed to monitor loss of mitochondrial membrane potential (ΔΨm). Values correspond to the percentage of cells exhibiting low uptake of DiOC₆. Results represent the means ± S.D. for three separate experiments performed in triplicate. * = significantly greater than values for LAMA-R cells exposed to 5 µM VP-16 alone (P < 0.01).
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