Molecular Characterization and Sensitivity of STI-571 (Imatinib Mesylate, Gleevec)-resistant, Bcr-Abl-positive, Human Acute Leukemia Cells to SRC Kinase Inhibitor PD180970 and 17-Allylamino-17-demethoxygeldanamycin

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ABSTRACT

Using human acute leukemia HL-60/Bcr-Abl (with ectopic expression of p185 Bcr-Abl) and K562 cells (with endogenous expression of p210 Bcr-Abl) subjected to a continuous selection pressure of up to 1.0 μM Gleevec (imatinib mesylate, STI-571), we have isolated Gleevec-resistant K562 R (+Bcr-Abl), K562 R (−Bcr-Abl), and HL-60/Bcr-Abl R cells, which display disparate level and activity of Bcr-Abl tyrosine kinase (TK). As compared with their sensitive counterparts, Gleevec-resistant cell types were ≥5-fold resistant to Gleevec-induced apoptosis. Bcr-Abl protein levels were significantly increased in HL-60/Bcr-Abl R and K562 R (+Bcr-Abl) cells, but K562 R (−Bcr-Abl) cells showed a marked decline in the mRNA and protein levels and activity of Bcr-Abl. Bcr-Abl TK level and activity corresponded to the signal transducers and activators of transcription-5 DNA binding activity and up-regulation of heat shock protein 70 levels. The decline in Bcr-Abl expression and TK activity in K562 R (−Bcr-Abl) cells was associated with reduced AKT kinase and signal transducers and activators of transcription-5 DNA binding activities and increased sensitivity to the death ligand Apo-2 ligand/tumor necrosis factor-related apoptosis-inducing ligand and 1-β,1-arabino-furanosylcytosine-induced apoptosis. All Gleevec-resistant cell types were sensitive to 17-allylamino-17-demethoxygeldanamycin (17-AAG)- and PD180970 (a SRC and Bcr-Abl TK inhibitor)-induced apoptosis. Treatment with 17-AAG or PD180970 also induced apoptosis of CD34+ leukemia cells from three patients with chronic myeloid leukemia in blast crisis who had progressive leukemia while receiving Gleevec therapy. Taken together, these findings indicate that in addition to overexpression or mutations in Bcr-Abl, resistance to Gleevec may also develop due to a loss of Bcr-Abl expression. These findings also support the rationale to test the in vivo efficacy of 17-AAG and PD180970 against STI-571-resistant Bcr-Abl-positive acute leukemias.

INTRODUCTION

The dysregulated activity of the TK encoded by bcr-abl, a human fusion oncogene, is an important contributor toward the malignant phenotype of Bcr-Abl-expressing CML-BC and ALL cells (1–4). Leukemic blasts from CML-BC and Bcr-Abl-positive ALL display differentiation arrest and resistance to apoptosis, even after exposure to high doses of antileukemic drugs (5–7). Several diverse molecular mechanisms known to inhibit apoptosis and confer drug resistance are constitutively active in Bcr-Abl-positive acute leukemia cells. For example, Bcr-Abl TK activity leads to phosphorylation and increased transactivation by STAT-5, resulting in increased expression of the antiapoptotic Bcl-xL protein (8, 9). In addition to STAT-5, Bcr-Abl TK induces the phosphatidylinositol 3′-kinase/AKT kinase and nuclear factor-κB activities, which are known to inhibit apoptosis by several mechanisms (10–15). Hence, targeting Bcr-Abl TK activity is a rational strategy for treating CML and Bcr-Abl-positive ALL (16–18).

Imatinib mesylate (Gleevec), previously known as STI-571, is a phenylaminopyrimidine derivative that is a relatively specific, ATP-binding site antagonist of Bcr-Abl, platelet-derived growth factor receptor and c-Kit TKs (19–22). At relatively low and clinically achievable concentrations (~0.25 μM), Gleevec selectively induces growth arrest and apoptosis of Bcr-Abl-positive leukemia cells, but not of normal hematopoietic progenitor cells (21, 22). Gleevec inhibits Bcr-Abl TK activity and induces apoptosis of HL-60/Bcr-Abl (with ectopic expression of p185 Bcr-Abl) and K562 cells (with endogenous expression of p210 Bcr-Abl; Ref. 23). This was associated with down-regulation of Bcl-xL levels and AKT kinase activity (23). Cotreatment with Gleevec also sensitized HL60/Bcr-Abl and K562 cells to Ara-C, etoposide-, or doxorubicin-induced apoptosis (23–25). Recent studies from our laboratory have shown that Bcr-Abl expression is also associated with resistance against Apo-2L/TRAIL-induced cytosolic accumulation of cytochrome c, processing and activation of caspase-9 and caspase-3, and apoptosis of HL60/Bcr-Abl and K562 cells (26–28). Cotreatment with Gleevec significantly increased Apo-2L/TRAIL-induced apoptosis of HL-60/Bcr-Abl and K562 cells (28). In addition to Gleevec, PD180970, which was originally shown to be a SRC TK inhibitor, also potently inhibited Bcr-Abl TK activity and induced apoptosis of K562 cells (29–30). Recently, the inhibitors of Hsp90 such as geldanamycin, or its analogue, 17-AAG, have been shown to disrupt the chaperone association of Bcr-Abl TK with Hsp90, promote the proteasomal degradation of Bcr-Abl, and induce differentiation and apoptosis of HL-60/Bcr-Abl and K562 cells (31).

Although treatment with Gleevec achieves complete hematological remission in >95% of patients with CML, complete cytogenetic and molecular responses are observed in only a minority of patients (32). Complete remissions are also observed in patients with myeloid or lymphoid blast crisis of CML or Philadelphia chromosome-positive ALL (33), but most patients enjoy only a short duration of response, with eventual emergence of Gleevec-resistant leukemic cells and a clinical relapse (34). To understand the possible mechanism of resistance to STI-571, several groups have recently reported the isolation of Gleevec-resistant, Bcr-Abl-positive, human acute leukemia cells that were selected for resistance after prolonged culture in progressively higher concentrations of Gleevec (35–37). Analyses of these cell lines have shown several mechanisms of resistance to Gleevec. These have included amplification of the bcr-abl gene, increased expression of the Bcr-Abl protein without amplification of the gene, or increased expression of the MDR-1 gene-encoded PGP (35–37). Human CML-BC
cells growing in mice have also been shown to acquire resistance to STI-571, based on increased serum levels of the e1 acetylcoenzyme A carboxylase that binds and blocks the uptake and activity of STI-571 in Bcr-Abl-positive CML-BC cells (38). Recently, biochemical and molecular analyses of leukemic cells from patients with CML-BC showed that acquired resistance to STI-571 was associated with reactivation of the Bcr-Abl TK (39). This was either due to a point mutation in the kinase domain of Bcr-Abl or amplification of the bcr-abl gene (39, 40). However, strategies that would exert cytotoxic effects against Gleevec-resistant cells have not been reported. In the present studies, we isolated Gleevec-resistant HL-60/Bcr-Abl R and K562 R (+Bcr/ Abl) cells that showed increased levels of Bcr-Abl protein. We have also isolated K562 R (=Bcr/Abl) cells, which contain markedly decreased mRNA and protein expression of Bcr-Abl, a heretofore unreported finding in Gleevec-resistant leukemic cells selected under the continuous presence of Gleevec. Importantly, we demonstrate for the first time that regardless of Bcr-Abl expression, HL-60/Bcr-Abl R as well as K562 R cells remain highly sensitive to apoptosis induced by PD180970 and 17-AAG. In addition, we demonstrate that leukemic blasts harvested from the bone marrow of three patients with CML-BC who demonstrated leukemia progression while receiving Gleevec were susceptible to PD180970- or 17-AAG-induced apoptosis.

MATERIALS AND METHODS

Reagents. Gleevec was a gift from Dr. Elisabeth Buchdunger of Novartis Pharma AG (Basel, Switzerland). Ara-C was purchased from Sigma Chemical Co. (St. Louis, MO) and prepared as a 10 mm stock solution in sterile PBS and diluted in RPMI 1640 before use. Gentec (South San Francisco, CA) kindly provided homotrimetric Apo-2L/TRAILE 17-AAG was obtained from the Developmental Therapeutics Branch of the Cancer Therapy Evaluation Program/ National Cancer Institute/NIH (Bethesda, MD). The pyridozo[2,3-d]pyrimidine derivative PD180970 was synthesized by Parke-Davis Pharmaceuticals and was kindly provided by Dr. Alan Kraker (29). PD180970 was diluted in DMSO. Anti-Bcl-2 antibody was purchased from DAKO (Carpinteria, CA), anti-Bcl-x<sub>L</sub> was purchased from PharMingen, Inc. (San Diego, CA), anti-Abl antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and 4E3 antibody was purchased from Signet Laboratories, Inc. (Bedford, MA). Protein G-agarose beads were washed twice in lysis buffer and then incubated with anti-Abl mAb (1:100 dilution; Santa Cruz Biotechnology) at 4°C for 2 h. After washing the Protein G beads and the antibody mix with lysis buffer, 100 μg of total cell lysates were added and incubated overnight at 4°C. The immunoprecipitates were washed three times in lysis buffer, and proteins were eluted with the SDS sample-loading buffer. Proteins were separated by SDS PAGE as described previously (42). Immunoprecipitates were examined by Western blot analysis after transfer of proteins to nitrocellulose membranes (42, 43). Western blot analyses were performed with phospho-tyrosine antibody (PharMingen) and anti-Abl antibody to control for equal loading (43). AKT Kinase Assay. AKT kinase activity was determined by using a phosphoimmunoprecipitation kinase assay with reagents provided in a commercially available kit (New England Biolabs, Beverly MA; Ref. 23). Briefly, cell lysates were used to immunoprecipitate AKT, using a polyclonal anti-AKT antibody. Immunoprecipitates were then incubated with GSK-3α/β fusion protein in the presence of ATP and kinase buffer, allowing immunoprecipitated AKT to phosphorylate GSK-3α, which was analyzed by Western blotting using an anti-phospho-GSK-3α/β (serine 21/9) antibody (23). Electrophoretic Mobility Shift Analysis for STAT-5. A previously described method was used (44). The cells were lysed in buffer A [10 mm HEPES (pH 7.9), 1.5 mm MgCl<sub>2</sub>, 10 mm KCl, 0.5 mm DTT, and 0.5 mm PMSF]. Cell lysates were left on ice for 10 min and then centrifuged at 12,000 × g for 30 s. Pellets were resuspended in buffer A containing 0.05% NP40. After 20 strokes of B pestle-tight fit to release the nuclei, the nuclei were then pelleted by centrifugation at 12,000 × g for 10 min. Nuclei were resuspended in buffer C [5 mm HEPES (pH 7.9), 26% glycerol, 1.5 mm MgCl<sub>2</sub>, 0.2 mm EDTA, 0.5 mm DTT, 0.5 mm PMSF, 0.3 mm NaCl], mixed well (~10 strokes), and left on ice for 30 min. Finally, nuclear extracts were collected as the supernatant after centrifugation at 12,000 × g for 30 min at 4°C. The nuclear extracts (~45 μg) were incubated with 2.0 μg of poly(dI-dC) for 15 min on ice, followed by 15 min of incubation with 1.0 ng of Klenow-labeled DNA harboring the STAT-5 optimal double-stranded DNA binding sequence 5′-GATCCGAGTTCCGGAATTCA-3′. For supershift analysis, before the addition of poly(dI-dC), the
nuclear extracts were incubated with anti-STAT-5 antibody (R&D Systems) or mouse IgG as a control, for 30 min on ice. DNA-protein complexes were separated on a 5% nondenaturing polyacrylamide gel in 22× Tris-borate EDTA (1× Tris-borate EDTA = 50 mM Tris-borate and 1.0 mM EDTA) and analyzed by autoradiography.

**FISH Analysis of the bcr-abl Gene.** Cells suspensions were cytospun onto slides, pre-aged with 2× SSC, and codenatured with DNA probe to 72°C for 2 min. Slides were treated with the dual-color bcr-abl translocation probe (Vysis, Inc., Downers Grove, IL) and hybridized for 20 h at 37°C. Slides were then washed with 0.4× SSC at 70°C for 3 min, followed by two washes with 2× SSC/0.1% NP40 at room temperature for 1 min. After rinsing once with PBS, slides were counterstained with 4',6-diamidino-2-phenylindole. Analyses were performed by an Olympus BX 40 fluorescence microscope using 82000 Series fluorescence filters. Two hundred interphase or metaphase cells were counted.

**Real-Time Quantitative RT-PCR.** Total RNA was isolated from cells using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Two μl of total RNA (0.1 μg) were added to 23 μl of a reaction mix (Taquin RT-PCR reagent kit; Perkin-Elmer, Boston, MA) consisting of 19.7 μl of H2O, 0.15 μl of enzyme mix, 0.5 μl of the forward B2A2 primer, 0.5 μl of reverse B2A2 primer, 1.0 μl of bcr-abl gene-specific oligonucleotide probe, 0.5 μl of histone H1 forward primer, 0.5 μl of histone H1 reverse primer, and 0.5 μl of histone-specific oligonucleotide probe and placed in a 96-well plate. The plate was transferred to an ABI 7700 thermocycler (Perkin-Elmer), and samples were passed through 48°C for 30 min and 95°C for 10 min, followed by 35 amplification cycles (95°C for 15 s and 60°C for 1 min), during which fluorescent signals of bcr-abl and histone H1 were measured as described previously (45). Serial dilutions of 10³ to 10⁴ of bcr-abl plasmid DNA and histone plasmid DNA were used as a reference standard of fluorescent signals for copy number, as described previously (45). Histone H1 primers (forward primer, 5′-GGGAGAATTCGCTACGATTATA-3′; reverse primer, 5′-GTGTCTTCAAAAGGCCAACCA-3′; and probe 5′-CCATCTGGCCCTTTGCAAGGCAC-3′) were used in the reaction. Other primers were as follows: (a) p210 Bcr-Abl (e2a2), forward primer 5′-TCACTCAGCAGCTGTTA-3′, reverse primer 5′-TGGAGCTCAAGTCTAATGTTA-3′, and probe CAGAGTTCAAAAGCCCTTCAGCGG; and (b) p185 Bcr-Abl (e1a2), forward primer 5′-CTGGAACCTCCAGCTTAC-3′, reverse primer 5′-AAAGTGACATTACGATCCGGG-3′, and probe 5′-TGACCCATCCTGGAGAAGCCTTCACCACG-3′. All of the probes carried a 5′-6-carboxy fluorescein reporter label and a 3′-6-carboxy-tetramethyl rhodamine quencher group. All primers and probes were synthesized by PE-Applied Biosystems (Perkin-Elmer).

**Apoptosis Assessment by Annexin V Staining.** After drug treatments, cells were resuspended in 100 μl of the staining solution (containing Annexin V fluorescein and propidium iodide in a HEPES buffer; Annexin V-FLUOS Staining Kit; Boehringer Mannheim, Indianapolis, IN). After incubation at room temperature for 15 min, cells were analyzed by flow cytometry (46). Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of those cells that have a compromised cell membrane. This allows for the discrimination of live cells (unstained with either fluorochrome) from apoptotic cells (stained only with Annexin V) and necrotic cells (stained with both Annexin V and propidium iodide; Ref. 46).

**Morphology of Apoptotic Cells.** After drug treatment, 50 × 10⁴ cells were washed and resuspended in PBS (pH 7.3). Cytospan preparations of the cell suspensions were fixed and stained with Wright stain. Cell morphology was determined by light microscopy. In all, five different fields were randomly selected for counting of at least 500 cells. The percentage of apoptotic cells was calculated for each experiment, as described previously (6).

**Statistical Analysis.** Significant differences between values obtained in a population of leukemic cells treated with different experimental conditions were determined using the Student t test.

**RESULTS**

**Isolation of Gleevec-resistant HL-60/Bcr-Abl and K562 Cells.** We have previously reported on the differentiation and apoptotic effects of Gleevec on HL-60/Bcr-Abl and K562 cells. To determine the potential mechanisms of resistance to Gleevec and evaluate novel strategies against Gleevec-resistant cells, we generated Gleevec-resistant HL-60/Bcr-Abl and K562 cells. HL-60/Bcr-Abl and K562 cells were cultured in the continuous presence of progressively higher levels of Gleevec, as described above. We isolated HL-60/Bcr-Abl and K562 cells that are capable of continuous growth in 1.0 μM Gleevec. However, the findings presented here were derived from studies performed on Gleevec-resistant HL-60/Bcr-Abl R cells that show unimpaired growth in 0.4 μM Gleevec or were derived from Gleevec-resistant K562 cells that exhibit growth in 1.0 μM Gleevec, i.e., K562 R (+Bcr-Abl) or K562 R (−Bcr-Abl) cells. Fig. 1, A and B, shows that exposure to 0.5 μM Gleevec for 48 h induced apoptosis of approximately 48% of HL-60/Bcr-Abl cells but of <10% of HL-60/Bcr-Abl R cells. After treatment with higher levels of Gleevec, i.e., 1.0 or 1.5 μM, apoptosis was observed in approximately 70% of HL-60/Bcr-Abl cells, as compared with 32% and 48% of HL-60/Bcr-Abl R cells, respectively. In contrast, K562 R (−Bcr-Abl) cells were markedly resistant to Gleevec-induced apoptosis, with <10% of the cells showing apoptosis after exposure to up to 5.0 μM Gleevec. K562 R (+Bcr-Abl) showed a modest dose-dependent sensitivity to higher doses of Gleevec, with 18% and 35% of the cells showing apoptosis after treatment for 48 h with 2.0 and 5.0 μM Gleevec, respectively (Fig. 1B).

**Molecular Correlates of Gleevec Resistance in HL-60/Bcr-Abl R and K562 R Cells.** To determine the molecular mechanisms of Gleevec resistance, we compared the protein and mRNA levels and

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Fig. 1. STI-571-induced apoptosis of STI-571-sensitive or STI-571-resistant HL-60/Bcr-Abl or K562 cells. Cells were washed and incubated with or without the indicated concentration of STI-571 for 72 h. After this, the percentage of apoptotic cells was determined by Annexin V staining and flow cytometry. Values represent the mean ± SD of three separate experiments. A, HL-60/Bcr-Abl versus HL-60/Bcr-Abl R cells; B, K562 versus K562 (+Bcr-Abl or −Bcr-Abl) cells.
autophosphorylation activity of Bcr-Abl TK among the Gleevec-sensitive and -resistant cell types. As compared with HL-60/Bcr-Abl and K562 cells, HL-60/Bcr-Abl R and K562 R (+Bcr-Abl) cells expressed approximately 4-fold higher levels of Bcr-Abl (Fig. 2A). However, the auto-tyrosine phosphorylation of Bcr-Abl was significantly increased (approximately 2-fold) only in K562 R (+Bcr-Abl) cells versus K562 cells (Fig. 2B). Despite higher expression of Bcr-Abl, autophosphorylation of Bcr-Abl in HL-60/Bcr-Abl R cells was similar to that in HL-60/Bcr-Abl cells (Fig. 2B). The levels of autophosphorylation of Bcr-Abl have been clearly correlated with its phospho-tyrosine activity and tyrosine phosphorylation of its downstream target proteins, such as CrkI or Gab-2 (30).

These data suggest that the increased levels of Bcr-Abl in HL-60/Bcr-Abl R cells may be kinase inactive. Treatment with up to 1.0 μM Gleevec did not affect the auto-tyrosine phosphorylation of HL-60/Bcr-Abl R and K562 R (+Bcr-Abl) cells (Fig. 2C). Both Bcr-Abl levels and its autophosphorylation were undetectable in K562 R (−Bcr-Abl) cells (Fig. 2, A and B). Next, we determined whether the increase or loss of expression of Bcr-Abl protein in the Gleevec-resistant cell types was due to an altered level of transcription of the fusion gene. Using a real-time RT-PCR assay, we determined the levels of bcr-abl mRNA in the Gleevec-sensitive and -resistant HL-60/Bcr-Abl and K562 cells. Table 1 shows that the bcr-abl/histone H1 mRNA copy number ratio in HL-60/Bcr-Abl R cells was similar to that in HL-60/Bcr-Abl cells, whereas it was approximately 3-fold higher in K562 R (+Bcr-Abl) cells versus K562 cells. However, it was markedly decreased in K562 R (−Bcr-Abl) cells versus K562 cells (Table 1). Furthermore, we also examined the copy number of the bcr-abl fusion gene by FISH analysis using separate bcr- and abl-specific probes. HL-60/Bcr-Abl R and K562 R (+Bcr-Abl) cells did not show bcr-abl gene amplification by the FISH analysis (data not shown). K562 R (−Bcr-Abl) cells showed a single copy of the bcr and abl genes. The bcr-abl fusion gene was not detectable by FISH analysis (data not shown). These findings indicate that despite the similar selection pressure under which the cells were cultured, quite disparate mRNA and protein levels of Bcr-Abl and its auto-tyrosine phosphorylation activity emerged in the Gleevec-resistant K562 R (−Bcr-Abl) cells versus K562 R (+Bcr-Abl) and HL-60/Bcr-Abl R cells.

We also compared the expression of other determinants of apoptosis in the Gleevec-sensitive and -resistant HL-60/Bcr-Abl and K562 cells. Results of the immunoblot analyses in Fig. 3 show that in the various cell types, the expression of Bcr-Abl was associated with increased Bcl-xL and Hsp70 expression. Conversely, Bcr-Abl expression in HL-60/Bcr-Abl and K562 cells was associated with down-regulation of Bcl-2 levels. The emergence of Gleevec resistance in HL-60/Bcr-Abl R, K562 R (−Bcr-Abl), and K562 R (+Bcr-Abl) cells was associated with a decline in Bcl-xL levels; Bcl-xL was undetectable in K562 R (−Bcr-Abl) cells. Undetectable levels of Bcr-Abl in

![Table 1: bcr-abl mRNA copy numbers in Gleevec-sensitive and -resistant HL-60/Bcr-Abl and K562 cells](image)

<table>
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<th>bcr-abl mRNA (×10^3 copies)</th>
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<th>bcr-abl/histone mRNA copy ratio</th>
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<td>K562 R (+Bcr-Abl)</td>
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<td>22.10</td>
<td>2.56</td>
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Fig. 3. Immunoblot analyses of the total cell lysates from HL-60/Bcr-Abl, HL-60/Bcr-Abl R, K562, and K562 R (+Bcr-Abl or −Bcr-Abl) cells. Cell lysates were immunoblotted with anti-Bcl-xL, anti-Bcl-2, anti-Hsp70, anti-Hsp90, anti-survivin, anti-c-IAP1, and anti-XIF antibodies (see "Materials and Methods"). β-Actin levels were used as a control for protein loading.

Fig. 2. A, immunoblot analyses of the total cell lysates from HL-60/Bcr-Abl, HL-60/Bcr-Abl R, K562, and K562 R (+Bcr-Abl or −Bcr-Abl) cells. Cell lysates were immunoblotted with anti-Abl antibody (see "Materials and Methods"). β-Actin levels were used as a control for protein loading. B, autophosphorylated Bcr-Abl protein levels were determined in the cell lysates from HL-60/Bcr-Abl, HL-60/Bcr-Abl R, K562, K562 R (+Bcr-Abl), and K562 R (−Bcr-Abl) cells, using protein G-agarose beads coated with anti-Abl antibody. Immunoprecipitates were obtained from cell lysates and immunoblotted with specific anti-phosphotyrosine antibody or anti-Abl antibody. C, Gleevec does not inhibit the autophosphorylation of Bcr-Abl TK in Gleevec-resistant cells. Immunoprecipitates were obtained as described in B from the indicated cell types after treatment with or without 1.0 μM Gleevec. These were immunoblotted with anti-phosphotyrosine or anti-Abl antibody.

![Fig. 2](image)
K562 R (−Bcr-Abl) cells were also associated with a loss of Hsp70 expression. Bcl-2 and XIAP expression increased with the emergence of Gleevec resistance in K562 cells but remained undetectable in HL-60/Bcr-Abl R cells (Fig. 3). No significant correlation was detected between Gleevec resistance and associated changes in Bcr-Abl levels with the expression of Hsp90, survivin, XIAP, or c-IAP1 proteins (Fig. 3) or the expression of Bax protein (data not shown).

Gleevec-resistant Cells Lack Overexpression of PGP or MRP-1. Because a previous report had noted an association of Gleevec resistance with expression of the MDR-1-encoded PGP, we estimated the cell membrane expression of PGP by flow cytometry in Gleevec-sensitive and -resistant cell types. Fig. 4 shows that, as compared with HL-60/Bcr-Abl and K562 cells, HL-60/Bcr-Abl R and K562 R (−Bcr-Abl) or K562 R (−Bcr-Abl) cells (data not shown) did not show increased PGP expression. 8226/Dox40 and HL-60/Neo cells were used as the positive and negative control for PGP expression, respectively (Fig. 4). Gleevec-resistant cell types also did not show an increase in MRP-1 expression by flow cytometric and Western analyses (data not shown).

AKT, SRC, and ERK1/2 Levels and STAT-5 Activity in Gleevec-resistant and -sensitive Cells. Previous reports had shown that Bcr-Abl TK activity leads to phosphorylation and increased transactivation by STAT-5, which induced the expression of anti-apoptotic Bcl-xL protein (8, 9). Bcr-Abl TK activity also increased the activity of phosphatidylinositol 3'-kinase/AKT kinase (10–12).

Hence, we analyzed the AKT kinase and STAT-5 activities in Gleevec-resistant and -sensitive cell types. As shown in Fig. 5, A and B, the STAT-5 and AKT kinase activities were increased in K562 and HL-60/Bcr-Abl cells versus HL-60/Neo cells, which is consistent with the previously reported observations. AKT kinase and STAT-5 activities were decreased in HL-60/Bcr-Abl R cells versus HL-60/Bcr-Abl and K562 R (−Bcr-Abl) cells versus K562 cells (Fig. 5, A and B). In contrast, K562 R (+Bcr-Abl) cells demonstrated increased AKT kinase (Fig. 5B) and STAT-5 activities (data not shown), as compared with K562 cells. This is consistent with the increased auto-tyrosine phosphorylation activity of Bcr-Abl TK in K562 R (+Bcr-Abl) cells.

We also determined the expression of p60 SRC and ERK1/2 kinase as well as phospho-ERK1/2 and phospho-SRC levels in HL-60/Bcr-Abl and K562 cells. Western analyses shown in Fig. 5 demonstrate that the decline in ERK1/2, SRC kinase, and phospho-SRC levels in K562 R (−Bcr-Abl) cells correlated with the loss of Bcr-Abl expression. In contrast, phospho-ERK1/2 levels increased with the loss of Bcr-Abl expression.
Activity of Ara-C and Novel Agents in Gleevec-resistant and -sensitive HL-60/Bcr-Abl and K562 Cells. We had previously demonstrated that HL-60/Bcr-Abl and K562 cells are relatively resistant to apoptotic stimuli that trigger the extrinsic and/or intrinsic pathway of apoptosis (7). Therefore, we determined the sensitivity of Gleevec-resistant HL-60/Bcr-Abl and K562 cells to Ara-C or Apo-2L/TRAIL-induced apoptosis. Table 2 shows that K562 R (+Bcr-Abl) cells are as sensitive as K562 cells to Ara-C or Apo-2L/TRAIL-induced apoptosis, whereas HL-60/Bcr-Abl R cells were slightly more sensitive than HL-60/Bcr-Abl cells to Apo-2L/TRAIL-induced apoptosis but not Ara-C-induced apoptosis. As compared with K562 cells, K562 R (−Bcr-Abl) cells were significantly more sensitive to both Ara-C- and Apo-2L/TRAIL-induced apoptosis, similar to AML cells that lack Bcr-Abl expression (7, 27).

Gleevec-resistant HL-60/Bcr-Abl and K562 or Patient-derived Leukemic Cells Are Sensitive to PD180970- and 17-AAG-induced Apoptosis. PD180970 is an ATP-competitive inhibitor of SRC and Bcr-Abl TKs, whereas 17-AAG down-regulates Bcr-Abl levels (30, 31). Both agents have been shown to induce apoptosis of K562 cells (30, 31). Therefore, we first determined the cytotoxic effects of these novel anti-Bcr-Abl agents against Gleevec-resistant HL-60/Bcr-Abl (30, 31). Both agents have been shown to induce apoptosis of K562 cells (30, 31). However, we have also demonstrated increasing leukemia blast counts in peripheral blood and bone marrow while receiving Gleevec therapy. Exposure to up to 1.0 μM Gleevec for 48 h did not increase apoptosis of these cells cultured in RPMI 1640 in 40% of the patient’s own serum (data not shown). Table 3 shows that, under similar culture conditions, leukemic cells from all of the patients were highly sensitive to 17-AAG. Except for the cells from patient 3, the leukemic cells were also highly sensitive to PD180970-induced apoptosis. Whereas 17-AAG has been shown to induce apoptosis, PD180970 is not active against Bcr-Abl-negative HL60 cells (30). Due to the limitations of the available sample size of the leukemic blasts, we were unable to verify the effects of PD180970 and 17-AAG on the Bcr-Abl TK activity and levels, respectively.

DISCUSSION

Although it yields clinical responses in a majority of patients with CML-BC and Bcr-Abl-positive ALL, resistance to Gleevec commonly develops, resulting in clinical relapse. This highlights a necessity to test novel strategies in the treatment of these types of leukemias (39, 40). Recent reports have shown that the resistance to Gleevec of leukemic blasts from patients who exhibit disease progression while receiving Gleevec therapy may be due to either a Bcr-Abl-dependent or Bcr-Abl-independent mechanism (34, 39). A Bcr-Abl-dependent mechanism, in which Bcr-Abl TK activity is retained, may be due to either a point mutation in the kinase domain of Bcr-Abl or amplification of bcr-abl gene. In addition, clinical resistance to Gleevec may also be due to a host response in which the leukemic cells would be expected to remain sensitive, ex vivo, to Gleevec, although this has not yet been demonstrated to occur in patients receiving Gleevec for Bcr-Abl-positive acute leukemias (38, 39). To elucidate cellular mechanisms of resistance to Gleevec, preclinical models of Bcr-Abl-positive human acute leukemia cell types have been created under the selection pressure of Gleevec. These have included cellular models that have Bcr-Abl overexpression with or without bcr-abl gene amplification and others that have shown overexpression of the MDR-1 gene-encoded PGP (36–37). Here, we report for the first time the isolation of two separate Gleevec-resistant K562 cell types and Gleevec-resistant HL-60/Bcr-Abl R cells: K562 R (+Bcr-Abl) and

Table 2. Ara-C- or Apo-2L/TRAIL-induced apoptosis of Gleevec-sensitive and -resistant HL-60/Bcr-Abl or K562 cells

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<tr>
<th>Cell Line</th>
<th>Control</th>
<th>Ara-C</th>
<th>Apo-2L/TRAIL</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562 R (−Bcr-Abl)</td>
<td>68 ± 0.5</td>
<td>64.0 ± 0.2</td>
<td>38 ± 0.2</td>
</tr>
<tr>
<td>K562 R (+Bcr-Abl)</td>
<td>82 ± 2.2</td>
<td>29.0 ± 2.1</td>
<td>48.8 ± 3.4</td>
</tr>
<tr>
<td>HL-60/Bcr-Abl R</td>
<td>68 ± 0.6</td>
<td>32.4 ± 1.2b</td>
<td>49.4 ± 0.8b</td>
</tr>
</tbody>
</table>

Table 3. PD180970- or 17-AAG-induced apoptosis of Gleevec-sensitive or -resistant K562 and HL-60/Bcr-Abl cells, as well as Bcr-Abl-positive leukemic blasts derived from three patients refractory to Gleevec therapy

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Control</th>
<th>1.0 μM</th>
<th>5.0 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>68 ± 0.5</td>
<td>22.2 ± 1.2</td>
<td>32.7 ± 2.5</td>
</tr>
<tr>
<td>K562 R (−Bcr-Abl)</td>
<td>64.0 ± 0.2</td>
<td>29.2 ± 3.4</td>
<td>44.2 ± 5.6</td>
</tr>
<tr>
<td>K562 R (+Bcr-Abl)</td>
<td>82 ± 2.2</td>
<td>250 ± 2.3</td>
<td>350.0 ± 2.8</td>
</tr>
<tr>
<td>HL-60/Bcr-Abl R</td>
<td>38 ± 0.2</td>
<td>29.0 ± 2.1</td>
<td>48.8 ± 3.4</td>
</tr>
<tr>
<td>HL-60/Bcr-Abl R</td>
<td>68 ± 0.6</td>
<td>32.4 ± 1.2b</td>
<td>49.4 ± 0.8b</td>
</tr>
</tbody>
</table>

Values are significantly different from those seen in 17-AAG-treated K562 cells.

Values are significantly different from those seen in the 17-AAG-treated HL-60/Bcr-Abl or K562 cells.

Table 3

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>0.1 μM</th>
<th>0.5 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>68 ± 0.5</td>
<td>22.2 ± 1.2</td>
</tr>
<tr>
<td>K562 R (−Bcr-Abl)</td>
<td>64.0 ± 0.2</td>
<td>29.2 ± 3.4</td>
</tr>
<tr>
<td>K562 R (+Bcr-Abl)</td>
<td>82 ± 2.2</td>
<td>250 ± 2.3</td>
</tr>
<tr>
<td>HL-60/Bcr-Abl R</td>
<td>38 ± 0.2</td>
<td>29.0 ± 2.1</td>
</tr>
<tr>
<td>HL-60/Bcr-Abl R</td>
<td>68 ± 0.6</td>
<td>32.4 ± 1.2b</td>
</tr>
</tbody>
</table>

Values are significantly different from those seen in 17-AAG-treated K562 cells.

Values are significantly different from those seen in the 17-AAG-treated HL-60/Bcr-Abl or K562 cells.
HL-60/Bcr-Abl R, which overexpress Bcr-Abl; and K562 R (–Bcr-Abl), which has a marked decline of bcr-abl mRNA and protein expression. K562 R (–Bcr-Abl) cells represent the first reported Gleevec-resistant leukemia cell type that has undetectable Bcr-Abl expression or Bcr-Abl TK activity. Thus far, this has not been reported as a mechanism of Gleevec resistance in leukemic blasts from patients demonstrating leukemia progression on Gleevec therapy. However, comprehensive evaluation of the mechanisms of resistance to Gleevec have not yet been determined from a large series of patients exhibiting Gleevec resistance. None of the Gleevec-resistant cell types demonstrated overexpression of PGP, as had been described for the Gleevec-resistant LAMA-84 cells (37). More importantly, regardless of their Bcr-Abl expression, all three Gleevec-resistant cell types were sensitive to apoptosis induced by PD180970, a TK inhibitor with different substrate specificity than Gleevec, and 17-AAG that lowers the levels of Bcr-Abl and other signaling kinases mediating survival signaling.

Whereas Bcr-Abl expression was increased both in K562 R (+Bcr-Abl) and HL-60/Bcr-Abl R cells, Bcr-Abl TK activity was only increased in K562 R (+Bcr-Abl) cells. This suggests that the increased Bcr-Abl expression in HL-60/Bcr-Abl R cells was not active as a TK. Also, because Gleevec treatment did not inhibit TK activity of Bcr-Abl in both K562 R (+Bcr-Abl) and HL-60/Bcr-Abl R cells, the mechanism of resistance to Gleevec in these cells appears to be Bcr-Abl dependent (34, 39). This would explain why these cells were sensitive to PD180970 and 17-AAG because these agents induce apoptosis by inhibiting survival signaling not only through Bcr-Abl but also through other mechanisms (29–31). On the other hand, Gleevec resistance of K562 R (–Bcr-Abl) cells was most likely due to a profound decline in Bcr-Abl expression to undetectable levels, which removed the dependency of these cells on Bcr-Abl-mediated survival signaling, thus making these cells unresponsive to Gleevec. It is well known that Gleevec is a relatively selective inhibitor of Bcr-Abl TK, sparing those cells that lack Bcr-Abl TK (21, 22). Despite the loss of Bcr-Abl, K562 R (–Bcr-Abl) cells have growth characteristics of leukemic blast in culture similar to those of CML-BC K562 cells (data not shown). Because, as noted above, PD180970 and 17-AAG can exert cytotoxic effects against human leukemic blast, regardless of the presence or lack of Bcr-Abl-mediated survival mechanisms, these agents retained activity against K562 R (–Bcr-Abl) cells. Decline in Bcr-Abl expression in K562 R (–Bcr-Abl) cells was also associated with the loss of the DNA binding activity of STAT5, as well as down-regulation of Hsp70 and Bcl-xL, as antiapoptotic mechanisms. In this context, it is noteworthy that Hsp70 is known to inhibit the intrinsic pathway of apoptosis by negatively regulating the Apaf-1-mediated apoptosome and late caspase-3-dependent apoptotic events (47, 48). As compared with the other cell types, K562 R (–Bcr-Abl) cells were relatively less sensitive to PD180970. This may be due to a decline in the SRC kinase combined with an increase in phospho-ERK1/2 levels in K562 R (–Bcr-Abl) cells. Up-regulation of ERK1/2 kinase activity has been shown to have antiapoptotic effects (49). In addition, the decline in Bcr-Abl TK in K562 R (–Bcr-Abl) cells was also associated with a down-regulation of AKT kinase activity, also known to exert antiapoptotic effects (10). As compared with K562 R (–Bcr-Abl) cells, HL-60/Bcr-Abl R cells, which have increased Bcr-Abl levels but not Bcr-Abl TK activity (Fig. 2, A and B), show a more profound decline in AKT kinase activity. This is difficult to explain, unless the kinase-inactive Bcr-Abl in these cells also has a dominant negative effect on Bcr-Abl auto-TK, leading to inhibition of AKT kinase.

Whether Gleevec resistance is Bcr-Abl dependent or Bcr-Abl independent is also important with respect to the sensitivity of these cells to apoptotic stimuli other than Gleevec. As reported previously and also shown here, Bcr-Abl expression confers resistance against Ara-C- and Apo-2L/TRAIL-induced apoptosis of HL-60/Bcr-Abl and K562 cells. Data presented here demonstrate that K562 R (+Bcr-Abl) and HL-60/Bcr-Abl R cells that, similar to HL-60/Bcr-Abl and K562 cells, retain Bcr-Abl expression and TK activity continue to show resistance to Ara-C- and Apo-2L/TRAIL-induced apoptosis. In contrast, K562 R (–Bcr-Abl) cells that have lost Bcr-Abl expression and TK activity regained their sensitivity to Ara-C- and Apo-2L/TRAIL-induced apoptosis. These findings may support the rationale to incorporate these agents in strategies directed against Bcr-Abl-independent Gleevec-resistant cells.

In the three samples of leukemic blasts from patients who had progressive disease while receiving Gleevec in vitro, resistance to 1.0 μM Gleevec was observed, making it unlikely that the resistance to Gleevec in these patients was solely due to host factors. In these patient-derived leukemic blasts, resistance to Gleevec may be a Bcr-Abl-dependent mechanism of resistance to Gleevec, although the

Fig. 6. Immunoblot analysis of PD180970- or 17-AAG-induced down-regulation of Bcr-Abl tyrosine kinase activity and protein levels of the HL-60/Bcr-Abl, HL-60/Bcr-Abl R, K562 S, K562 R (+Bcr-Abl), and K562 R (–Bcr-Abl) cells. A, HL-60/Bcr-Abl, HL-60/Bcr-Abl R, K562 S, K562 R (–Bcr-Abl), and K562 R (+Bcr-Abl) cells were incubated with or without 2.0 μM 17-AAG for 48 h. After this treatment, total cell lysates were analyzed for Bcr-Abl and Hsp70 protein levels by immunoblot analysis. B, HL-60/Bcr-Abl, HL-60/Bcr-Abl R, K562 S, K562 R (–Bcr-Abl), and K562 R (+Bcr-Abl) cells were incubated with 0.5 μM PD180970 for 24 h, and cell lysates were used to determine phosphorylated Bcr-Abl protein levels. Using protein G-agrose beads coated with anti-Abl antibody, Bcr-Abl was immunoprecipitated from cell lysates. Immunoprecipitates were then immunoblotted with specific anti-phosphory turbine of Bcr-Abl expression or anti-Bcr-Abl antibody.
existence of Bcr-Abl-independent mechanisms of Gleevec resistance cannot be excluded. Regardless of the mechanism responsible for Gleevec resistance, sensitivity of these leukemic cells to PD180970- and 17-AAG-induced apoptosis suggests that the intrinsic resistance to Gleevec can be overcome. It is clearly important to investigate whether 17-AAG would also be active in lowering the levels of Bcr-Abl possessing a kinase domain mutation as well as active in inducing apoptosis of these cells (39, 40). Similarly, it remains to be seen whether PD180970 would be active in Gleevec-resistant cells as an inhibitor of mutant Bcr-Abl or SRC TK (50, 51), thus overcoming Gleevec resistance. Collectively, these findings create a strong rationale to investigate the in vivo activity of these novel agents in Gleevec-sensitive and -resistant cellular models of Bcr-Abl-positive acute leukemias.

REFERENCES


Molecular Characterization and Sensitivity of STI-571 (Imatinib Mesylate, Gleevec)-resistant, Bcr-Abl-positive, Human Acute Leukemia Cells to SRC Kinase Inhibitor PD180970 and 17-Allylamino-17-demethoxygeldanamycin

Ramadevi Nimmanapalli, Erica O'Bryan, Mei Huang, et al.