Arsenic Trioxide Inhibits Translation of mRNA of bcr-abl, Resulting in Attenuation of Bcr-Abl Levels and Apoptosis of Human Leukemia Cells

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ABSTRACT

Present studies demonstrate that treatment with arsenic trioxide (AT) lowered ectopically expressed or endogenous levels of Bcr-Abl protein, as well as induced apoptosis of Bcr-Abl-expressing cultured and primary chronic myeloid leukemia cells, including those refractory to imatinib mesylate. Treatment with AT neither affected bcr-abl mRNA transcript levels nor promoted the proteasomal degradation of Bcr-Abl. Importantly, in [35S]methionine-labeled leukemia cells, exposure to AT rapidly lowered the levels of the newly synthesized Bcr-Abl, indicating inhibition of bcr-abl mRNA translation. Treatment with AT rapidly inhibited the activity of 3-phosphoinositide-dependent protein kinase-1, as well as levels of p70 S6 kinase. p70 S6 kinase-1 is known to be a positive regulator of the translation of a group of mRNAs that possesses a long and highly structured 5′-untranslated region (UTR) containing a tract of oligopyrimidines (TOP). Because bcr-abl mRNA was discovered to possess a long and highly structured 5′-UTR containing a 12-pyrimidine TOP sequence in its 5′-UTR, we determined the effect of AT in Jurkat cells with ectopic expression of a 5′-UTR-deleted mutant of the bcr-abl gene, i.e., Jurkat/Bcr-Abl (5′UTR-) cells. Treatment with AT neither lowered the levels of the 5′-UTR-deleted mutant of Bcr-Abl nor induced apoptosis of Jurkat/Bcr-Abl (5′UTR-) cells. Taken together, these findings demonstrate a novel mechanism by which AT down-regulates Bcr-Abl levels and induces apoptosis of Bcr-Abl-positive chronic myelogenous leukemia cells.

INTRODUCTION

The bcr-abl fusion oncogene is the molecular hallmark of 95% of CML3 and ~20% of adult ALL (1). In this oncogene, the c-abl gene is under the transcriptional control of the bcr locus (2). The mRNA of bcr-abl encodes for two main types of oncoproteins, i.e., the p210 and p185 Bcr-Abl TKs, found in CML and ALL, respectively (1). The constitutive and dysregulated activity of the Bcr-Abl TK in the cytosol activates several molecular mechanisms known to inhibit apoptosis, which may contribute toward drug resistance of Bcr-Abl-positive leukemia blasts (3–7). These mechanisms include increased transcriptional activities of STAT5 and nuclear factor-κB, which are known to induce the expression of the antiapoptotic Bcl-2 and IAP family of proteins (4, 5, 7). Bcr-Abl TK also activates the P38K/Akt kinases, which inhibits apoptosis through several known mechanisms (8–10). Preclinical in vitro and animal studies have demonstrated that specific targeting of the bcr-abl gene product exerts an antileukemia effect against Bcr-Abl-expressing leukemia cells (11–13). In the past, several treatment strategies that reduced the intracellular levels of the mRNA and/or protein levels of Bcr-Abl were shown to inhibit proliferation and clonogenic survival of Bcr-Abl-expressing leukemia cells (11, 13). Recently, treatment with the Bcr-Abl TK inhibitor, imatinib mesylate (Gleevec, formerly known as STI-571 or CPG57148B), has been shown to selectively inhibit the growth and induce apoptosis of leukemia cells that possess Bcr-Abl TK activity (14, 15). Although imatinib has impressive clinical activity against chronic phase CML, in the accelerated and blastic phases of CML (CML-BC) or Bcr-Abl-positive ALL, the clinical outcome after treatment with imatinib is unacceptably poor (16–18). This emphasizes the need to develop and test novel anti-Bcr-Abl strategies alone or in combination with imatinib in the advanced stages of CML.

AT (or Trisenox) is a highly active agent against APL (19). Treatment with AT down-regulates intracellular PML–RARα protein levels and induces differentiation and apoptosis of APL cells (20, 21). Recent reports have demonstrated that clinically achievable concentrations and schedules of AT markedly reduced the intracellular levels of Bcr-Abl and induced apoptosis of Bcr-Abl-positive acute leukemia cells (22, 23). In addition, the combined treatment with AT and imatinib was shown to exert superior apoptotic effects than either agent alone (23, 24). How ever, the mechanism by which AT reduces the intracellular Bcr-Abl levels, resulting in apoptosis of the highly drug-refractory, Bcr-Abl-positive acute leukemia cells, has not been investigated. In the present studies, we demonstrate that treatment with AT neither inhibits the mRNA transcript levels nor promotes proteasomal degradation of Bcr-Abl. Instead, exposure to AT rapidly inhibits the activity of PDK1, as well as lowers the in vitro and intracellular activity of the S6K1. This inhibits the translation of Bcr-Abl mRNA. The mRNA of Bcr-Abl was discovered to possess a long and structured 5′-UTR containing a characteristic 5′-terminal TOP. This 5′-UTR is demonstrated here to be required for the inhibition of Bcr-Abl mRNA translation by AT through inhibition of PDK1 and S6K1 activities, because AT did not reduce the levels of the Bcr-Abl encoded by an ectopically expressed 5′-UTR deletion mutant of the bcr-abl gene. Additionally, treatment with AT lowered Bcr-Abl levels and induced apoptosis of imatinib refractory, CD34+ primary CML-BC cells.

MATERIALS AND METHODS

Reagents. AT and cytarabine were purchased from Sigma (St. Louis, MO). AT was diluted and stocked as described previously (22). Whereas STI-571 (imatinib mesylate) was kindly provided by Dr. Elisabeth Buchdunger (Novartis Pharma, Basel, Switzerland), 17-AAG and PS-341 were obtained from sources described previously (25, 26). Antibodies for the immunoblot analyses to detect the levels of proteins including Bcl-xL, AKT, and B-catenin were obtained as described previously (15, 23).

Cells. CML-BC K562, LAMA-84, Jurkat/Bcr-Abl (wt), Jurkat/Bcr-Abl (TOP–), as well as HL-60/Bcr-Abl (wt) and HL-60/Bcr-Abl (T315I) cells were cultured and passaged, as described previously (15, 27). Bone marrow and/or peripheral blood samples from five patients with Bcr-Abl-positive CML-BC who had relapsed while receiving imatinib mesylate (Gleevec) up to

Received 5/19/03; revised 8/11/03; accepted 8/26/03. (Grant support: NIH/National Cancer Institute RO1s CA 090717 and CA 95188. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. Requests for reprints: Kapil Bhalla, Interdisciplinary Oncology Program, Moffitt Cancer Center, University of South Florida, 12900 Magnolia Drive, MRC 3 East, Room 3056, Tampa, FL 33612. Phone: (813) 903-6801; Fax: (813) 903-6817; E-mail: bhallak@moffitt.usf.edu. The abbreviations used are: CML, chronic myelogenous leukemia; CML-BC, CML blast crisis; ALL, acute lymphocytic leukemia; TK, tyrosine kinase; STAT, signal transducer and activator of transcription; AT, arsenic trioxide; APL, acute promyelocytic leukemia; S6K, p70 S6 kinase; UTR, untranslated region; TOP, tract of oligopyrimidines; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; wt, wild type; GSK-3α, glycogen synthase kinase-3α; cyt c, cytochrome c; RT-PCR, reverse transcription-PCR; Ara-C, 1-β-d-arabinofuranosylcytosine; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PDK1, 3-phosphoinositide-dependent protein kinase-1.
methionine-free medium, followed by incubation in medium containing 250 U/ml of antibody, as described previously (26).

Western Blot Analyses of Proteins. Western blot analyses of Bcr-Abl, Bcl-x<sub>L</sub>, AKT, S6K1, 4E-BP1, phospho-S6K1, phospho-4E-BP1, S6 protein and β-actin were performed as described previously (3, 22).

Immunoprecipitation and Autophosphorylation of Bcr-Abl. Untreated and drug-treated cells were lysed; immunoprecipitates containing Bcr-Abl were obtained from the cell lysates, as described previously (28). After protein transfer to membranes, Western blot analysis was performed using an anti-phosphotyrosine antibody (PharMingen, Inc., San Diego, CA; Ref. 28).

AKT Activity Assay. AKT kinase activity was determined by using an immunoprecipitation kinase assay with reagents provided in a commercially available kit (New England Biolab, Beverly, MA; Ref. 28). Briefly, cell lysates were used to immunoprecipitate AKT, using a polyclonal anti-AKT antibody. Immunoprecipitates were then incubated with GSK-3α 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, as described previously (15, 28).

Preparation of S-100 Fraction and Western Analysis of cyt c. Untreated and drug-treated cells were harvested, cytotoxic S-100 fraction was extracted, and Western blot analysis of cyt c was performed, as described previously (3).

Apgoptosis Assessment by Annexin-V Staining and Morphology. Untreated and drug-treated cells were washed and stained with Annexin-V and propidium iodide, and the percentage of apoptotic cells was determined by flow cytometry as described previously (15, 28). The morphological assessment of apoptosis was determined, as described previously (28).

Real-Time Quantitative RT-PCR Analysis of bcr-abl mRNA. Total RNA was isolated with an RNaseasy kit (Qiagen), reverse transcribed, and amplified using the reaction mixture in the Taqman RT-PCR reagent kit and ABI 7700 thermocycler from Perkin-Elmer (Boston, MA), as described previously (29). Histone H1 primers, reverse primer, and a probe as well as the p210 bcr-abl (bs3A2) forward primer, reverse primer, and a probe were used in the reaction. All primers and probes were synthesized by PE-Applied Biosystems (Perkin-Elmer; Refs. 28, 29).

Proteasomal Degradation of Bcr-Abl in NP40-soluble and -insoluble Protein Fractions. K562 cells were treated with AT and/or PS 341 or 17-AAG and/or PS 341. Untreated and drug-treated cells were lysed in the NP-40-containing lysis buffer, NP40-insoluble proteins were solubilized with SDS-containing buffer, and NP40-insoluble and soluble protein fractions were separated by 7.5% SDS-PAGE-containing buffer, and NP40-insoluble and soluble protein fractions were separated by 7.5% SDS-PAGE, and extracts were immunoprecipitated with a monoclonal anti-Abl monoclonal antibody, as described previously (26).

Bcr-Abl Translational-Inhibition Studies. Cells were cultured for 3 h in methionine-free medium, followed by incubation in medium containing 250 μCi/ml of [35S]methionine (Life Science Products, Boston, MA) in the presence or absence of 2.0 μM AT. After specific exposure intervals, cells were lysed in 1% NP40-containing buffer, and extracts were immunoprecipitated with a monoclonal anti-Abl antibody, as described above. In addition, the half-life of newly synthesized Bcr-Abl was determined, as described previously (30).

Intracellular and in Vitro Kinase Activities of S6 Kinase and Intracellular PDK1 Activity Assays. Cell lysates of untreated and drug-treated cells were used to immunoprecipitate S6K1 or PDK1, using a rabbit polyclonal anti-S6K1 or anti-PDK1 antibody (Cell Signaling Technology, Beverly, MA). The in vitro kinase assays were performed with the immunoprecipitates using an S6K1 or PDK1 assay kit (Upstate Biotechnology, Lake Placid, NY), as described previously (31, 32). The in vitro S6K1 activity assay was performed by using one molecule of the active recombinant S6K1 (Upstate Biotechnology) as the enzyme using the peptide substrate from the S6K1 assay kit, as described previously (31, 32).

Creation of Plasmid Expression Vectors and Transfectants. The bcr-abl cDNA was a kind gift of Dr. Thomas Skorski (Temple University, Philadelphia, PA). wt bcr-abl was cloned into the BamHI site of the plasmid vector pSTAR (27). To create the pSTAR bcr-abl (5′UTR)-expression vector, the wt bcr-abl plasmid was used as a template for PCR amplification using an upstream primer containing a BamHI site and a downstream primer containing the stop codon, followed by the EcoRI restriction site. The PCR product was digested with BamHI and EcoRI and subcloned into the pSTAR vector. The

p210 bcr-abl (T315I) mutant construct was created from the wt bcr-abl by site-directed mutagenesis, using the Quick-Change XL Mutagenesis kit (Clontech, Palo Alto, CA; Ref. 33). The resulting pSTAR bcr-abl (wt), p210 bcr-abl (T315I), and pSTAR bcr-abl (5′UTR)-plasmid vectors were stably transfected into Jurkat and HL-60 cells (27). Stable clones were selected, subcloned by limiting dilution method, and maintained, as described previously (27).

Statistical Analyses. Data were expressed as mean ± SE. Comparisons used Student’s t test or ANOVA, as appropriate. P of <0.05 was assigned significance.

RESULTS

Exposure to AT Down-Regulates Bcr-Abl Levels and Induces Apoptosis. We stably transfected the full-length cDNA of the bcr-abl (wt) gene that encodes for the p210 Bcr-Abl under a doxycycline-inducible promoter into Jurkat T leukemia cells (Jurkat/Bcr-Abl). Clones were isolated that, by immunoblot analysis, displayed doxycycline-inducible expression of Bcr-Abl. This was also associated with increased intracellular levels of Bcl-x<sub>L</sub> and AKT activity, the latter determined by assessing immunoprecipitated AKT to phospho-specificity of GST-3α, which was analyzed by Western blotting using an anti-phospho-GSK-3α/β (serine 21/9) antibody (Fig. 1A). AKT levels were not induced by doxycycline treatment of Jurkat/Bcr-Abl cells (data not shown). LAMA-84 and K562 cells that have a stable expression of p210 Bcr-Abl also possess abundant levels of Bcl-x<sub>L</sub> and AKT (Fig. 1B). These findings are consistent with the previous reports that by enhancing the phosphorylations and activities of STAT5 and PI3 kinase, Bcr-Abl TK increases the intracellular levels of Bcl-x<sub>L</sub> and AKT activity, respectively (9, 10). Cotreatment of Jurkat/Bcr-Abl cells with doxycycline and/or 5.0 μM AT for 48 h reduced the intracellular levels of Bcr-Abl and AKT activity (Fig. 1, A and B). Treatment of LAMA-84 and K562 cells with 2.0 or 5.0 μM AT for 48 h reduced the intracellular levels of Bcr-Abl and AKT, as well as inhibited AKT activity (Fig. 1B). Notably, at this dose and exposure interval, despite lowering Bcr-Abl, AT did not lower Bcl-x<sub>L</sub> expression (Fig. 1, A and B). This may be because STAT5 activity, as measured by electrophoretic mobility shift assay, was not reduced by exposure to 2.0 μM AT for 48 h (data not shown). This suggests that the effect of AT on Bcr-Abl is independent of its effect on the DNA-binding activity of STAT5. The mechanism underlying this differential effect of AT on Bcr-Abl versus STAT5 remains to be elucidated. Previous reports had demonstrated that Bcr-Abl-transformed cells are resistant to apoptosis induced by antileukemia drugs (3, 15) but succumb to apoptosis induced by AT and imatinib that lower Bcr-Abl levels and inhibit its TK activity, respectively (22, 23). Treatment with doxycycline, which induced the expression of Bcr-Abl, and AT induced apoptosis of Jurkat/Bcr-Abl cells, albeit significantly less than treatment with AT alone (P < 0.01; Fig. 1C). Thus, unlike the other antileukemia agents, e.g., Ara-C and etoposide, AT is able to induce apoptosis of Bcr-Abl-positive leukemia cells, only because it is able to lower the levels of Bcr-Abl along with delivering the apoptotic stimulus through its mitochondrial toxic effect. A dose-dependent increase in apoptosis was also observed after exposure of LAMA-84 cells to AT for 48 or 72 h (Fig. 1D).
levels in the insoluble fraction, which is known to contain misfolded proteins (Fig. 2, A and B; Ref. 34). This rules out the possibility that treatment with AT increases the proteasomal degradation of Bcr-Abl. In contrast, the decline in Bcr-Abl levels attributable to enhancement of its proteasomal degradation by 17-AAG, which is a heat shock protein 90 inhibitor, was restored by cotreatment with PS-341, as has been shown before (26). Another explanation for the decline in Bcr-Abl levels attributable to AT could be its inhibitory effect on the transcription, or of the mRNA half-life, of Bcr-Abl. After treatment of K562 cells with 2.0 μM AT, the ratios of bcr-abl to histone H1 mRNA transcript levels, as detected by a real-time RT-PCR assay (28, 29), remained essentially unaltered for 0, 24, or 48 h and were estimated to be 0.48, 0.44, and 0.46, respectively. Similarly, AT neither significantly altered the levels of bcr-abl mRNA transcript levels nor the ratio of bcr-abl to histone H1 mRNA levels in LAMA-84 cells (data not shown).

We next determined whether treatment with AT inhibits the mRNA translation of Bcr-Abl. After incubation in a methionine-free medium, K562 cells were cultured in [35 S]methionine-replete medium with or without 2.0 μM AT. Exposure to AT for intervals as short as 2 h markedly inhibited the levels of newly synthesized Bcr-Abl, without affecting Bcl-xL or AKT (Fig. 2, C and D). The translation of the ribosomal S6 protein was also inhibited. Alternatively, following a 3-h incubation of the cells in the [35 S]methionine-replete medium, as
ARSENIC TRIOXIDE INHIBITS Bcr-Abl TRANSLATION

Fig. 3. AT inhibits S6K1 and PDK1 activities and levels of p-S6 kinase. A, the amount of labeled phosphate incorporated by immunoprecipitated intracellular S6K1 into a substrate peptide of S6 protein, after the designated exposure intervals to 2.0 μM AT. B, AT-mediated inhibition of the amount of in vitro incorporation of labeled phosphate (represented as radioactivity) by recombinant S6K1 into a substrate peptide for S6 protein over 2 h. C, the effect of AT (2.0 or 5.0 μM for 2–6 h) on the amount of labeled phosphate incorporated by immunoprecipitated intracellular PDK1 into a substrate peptide. Bars, SD. D, effect of 2.0 μM AT and/or 100 ng/ml of rapamycin for 48 h on the intracellular levels of Bcr-Abl and p-S6 kinase in K562 cells. β-actin levels served as the loading control.

above, the cells were washed and incubated in the presence or absence of AT. The labeled Bcr-Abl levels were assessed to determine the AT effect on the half-life of Bcr-Abl. In K562 cells, the half-life of Bcr-Abl was only minimally shortened by treatment with AT and was approximately 12 h in both AT-untreated and treated cells (data not shown). Taken together, these studies clearly indicate that AT-mediated decline in Bcr-Abl levels was mainly attributable to inhibition of its protein translation, which was not due to a generalized effect of AT on protein translation. Thus, AT-mediated depletion of Bcr-Abl levels was neither attributable to decreased half-life of Bcr-Abl nor due to inhibition of its mRNA transcript levels.

AT Rapidly Inhibits PDK1 and S6K1 Activities and Causes Delayed Inhibition of 4E-BP1 Phosphorylation. Cap-dependent translation of most human mRNAs, including bcr-abl, is regulated mainly at the initiation (35, 36). The assembly of translation initiation complex at the mRNA cap and its translation is promoted by the activating phosphorylation of the ribosomal S6 protein by p70 S6K1 and/or the inactivating phosphorylation of 4E-BP1 by mTOR kinase. This allows 4E to participate, at the cap, in the translation initiation complex 4G (31, 35). S6K1 activity, through S6 phosphorylation, has also been shown to control the translation of 5′-TOP mRNAs, so named for the presence of an oligopyrimidine tract in their 5′-UTR, which is a characteristic of ribosomal proteins and elongation factors involved in the translation (37–39). Because AT inhibited the translation of Bcr-Abl and S6 protein, we determined the effect of treatment with AT on S6K1 phosphorylation and activity in K562 cells. Equal amounts of immunoprecipitates with specific S6K1 antibody were used to determine the S6K1 activity present in the untreated and AT-treated cells, which was measured by an in vitro S6K1 kinase assay (data not shown). Exposure to 2.0 μM AT for 4 h inhibited the intracellular S6K1 activity by ~70% (Fig. 3A). This rapid and early inhibition of S6K1 activity was associated with neither any significant decline in the phosphorylation and activity of AKT nor phosphorylation of 4E-BP1 (data not shown) but was coincident with the inhibition of Bcr-Abl translation by AT (Fig. 2C). However, total intracellular levels of Bcr-Abl declined noticeably (by ~30%) only after exposure to AT for ≥8 h (data not shown). Collectively, this discounted the possibility that the rapid inhibition of the intracellular S6K1 activity by AT was attributable to its inhibition of Bcr-Abl and/or AKT kinase. Treatment with 2.0 or 5.0 μM AT for 2–6 h inhibited the intracellular activity of PDK1 (Fig. 3C). To determine whether AT also inhibits S6K1 directly, we measured the effect of the in vitro exposure to AT on the activity of recombinant S6K1 to phosphorylate a substrate peptide in the presence of γ-ATP. Treatment with 2.0 or 5.0 μM AT partially inhibited the in vitro S6K1 activity within 2 h (Fig. 3B). Longer exposure intervals to 2.0 μM AT for 48 h also produced a decline in the levels and activity of AKT (Fig. 1B), as well as caused the down-regulation of phospho-S6K1 (Fig. 3D) and phospho-4E-BP1 in K562 cells (data not shown). Rapamycin is a known inhibitor of mTOR kinase, which phosphorylates and regulates the activity of S6K1 (35). Fig. 3D demonstrates that, although treatment with rapamycin for 48 h decreased the levels of phospho-S6K1, it did not lower Bcr-Abl levels in K562 cells. Also AT did not inhibit mTOR or phospho-mTOR levels in K562 cells (data not shown). Cotreatment with 100 ng/ml of rapamycin also did not enhance AT-mediated depletion of Bcr-Abl or phospho-S6K1 levels (Fig. 3D). These data also militate against the possibility of mTOR involvement in AT-mediated inhibition of S6K1 activity. Collectively, these data suggest that treatment with AT not only rapidly inhibits intracellular activity of S6K1 by attenuating PDK1 activity but also directly inhibits in vitro S6K1 activity modestly. This, in turn, inhibits Bcr-Abl translation and, consequently, depletes its TK activity. Downstream to inhibition of Bcr-Abl TK the activity of PI3K/AKT is inhibited, which would lead to the inhibition of the mTOR kinase-mediated phosphorylation of 4E-BP1 (9, 35, 37). These effects that follow down-regulation of Bcr-Abl because of longer exposure intervals to AT (48–72 h) may be responsible for attenuation of AKT levels and its kinase activity demonstrated in Fig. 1B. However, for this scenario to be true, the mRNA of Bcr-Abl would be expected to possess a long and structured 5′-UTR containing a characteristic 5′-terminal TOP, which would be susceptible to translation inhibition attributable to inhibition of PDK1 and S6K1 activities.

Bcr-Abl mRNA Has a TOP Sequence in Its 5′-UTR. The promoter of the human bcr gene regulates the transcription of the chimeric bcr-abl mRNA (2). Therefore, we searched for the putative TOP sequence in the 5′-UTR of the mRNA of the bcr-abl gene. A characteristic 12-pyrimidine tract, starting with a cytosine (C), and a C to thymidine (T) ratio of 8/4, was identified 455 nucleotides upstream to the ATG translation initiation site in the 5′-U5-nucleotide long 5′-UTR (GenBank accession number NM_021574; Fig. 4, A and B). 5′-TOP sequences have also been identified in the human mRNAs of other translation regulatory proteins, including the pol(A) binding protein and elongation factors 1A (heEF1A; Ref. 36). There is one more 11-pyrimidine-stretch TOP from bases 383 to 394 (although further away from the TTAA site) in the 5′-UTR of the mRNA of the bcr-abl gene. Could the long and highly structured 5′-UTR of the mRNA of bcr-abl containing the TOP sequences (Fig. 4C) have been responsible for the inhibition of translation of Bcr-Abl through AT-mediated attenuation of S6K1 activity? To confirm this, we cloned a deletoin-mutant construct of bcr-abl lacking the entire 5′-UTR (5′-UTR-Bcr-Abl) (Fig. 4D) into the pSTAR expression vector that has a doxycycline-inducible promoter (27). This, as well as the wt bcr-abl construct, was separately transfected into Jurkat cells. Subclones of the cells were isolated that demonstrated an inducible, albeit slightly leaky, expression of either wt or 5′UTR-Bcr-Abl when exposed to 5 μg/ml of doxycycline (Fig. 5A). Both the wt and 5′UTR-Bcr-Abl demonstrated the auto-tyrosine phosphorylation activity (Fig. 5B). As above, exposure to 2.0 μM AT for 48 h markedly reduced the intracellular levels of wt Bcr-Abl (Fig. 5A). Treatment with lower levels of...
AT (1.0 μM) had a lesser effect (data not shown). Importantly, exposure to 2.0 μM AT for 48 h failed to attenuate the levels of 5′UTR-Bcr-Abl (Fig. 5A), whereas treatment with 0.5 μM STI-571 for 48 h inhibited the auto-tyrosine phosphorylation activity of Bcr-Abl without lowering its levels (Fig. 5B). In Jurkat/Bcr-Abl (5′UTR-) cells, 2.0 μM AT plus doxycycline neither lowered AKT levels nor its activity (Fig. 5C) but reduced the phospho-S6K1 levels without lowering S6K1 and S6 protein levels (Fig. 5C). Treatment with 2.0 μM AT also induced the cytoplasmic accumulation of cyt c and apoptosis, more in the untreated than doxycycline-treated Jurkat/Bcr-Abl (wt) cells (Fig. 5, D and E). As shown in Fig. 5D, the inducible expression of Bcr-Abl partially reduced AT-induced apoptosis of Jurkat/Bcr-Abl (wt) cells. In contrast, AT-induced cytoplasmic accumulation of cyt c and apoptosis was significantly lower, following doxycycline-inducible expression of 5′UTR-Bcr-Abl in Jurkat/Bcr-Abl (5′UTR-) cells (P < 0.05; Fig. 5, D and E). In contrast to untreated cells, exposure to doxycycline inhibited Ara-C-induced apoptosis but sensitized Jurkat/Bcr-Abl (wt) cells to imatinib-induced apoptosis (Fig. 5F).

After exposure to doxycycline, Jurkat/Bcr-Abl (5′UTR-) cells displayed similar sensitivity to apoptosis to Ara-C and imatinib, as compared with Jurkat/Bcr-Abl (wt; Fig. 5F). This indicates that the auto-tyrosine phosphorylation, sensitivity to imatinib, and the ability to confer resistance to apoptosis because of Ara-C were retained by the 5′UTR-Bcr-Abl in Jurkat/Bcr-Abl (5′UTR-) cells. Collectively, these studies strongly support the contention that the presence of a long 5′UTR containing TOP sequences in the mRNA of bcr-abl is responsible for AT-mediated inhibition of the translation and attenuation of Bcr-Abl protein, which confers susceptibility to AT-induced apoptosis of Bcr-Abl-positive acute leukemia cells.

**Ex Vivo Exposure to AT Reduces Intracellular Levels of Bcr-Abl and Induces Apoptosis of Leukemia Blasts from Patients’ Refractory to Treatment with Imatinib.** We next determined whether, similar to the observations made in the cultured acute leukemia cells, exposure to AT would also down-regulate Bcr-Abl levels and induce apoptosis of primary CML-BC cells. For these studies, fresh samples of CD34+ leukemia progenitor cells were used, which were isolated from peripheral blood or bone marrow from five patients who had developed imatinib-refractory CML-BC. With three of these samples that yielded adequate numbers of leukemia progenitor cells, we determined the effect of AT on Bcr-Abl levels evaluated by immunoblot analysis. Exposure to 2.0 μM AT for 24 h produced a decline in Bcr-Abl levels to a variable extent in the three samples (Fig. 6A). In all of the five samples of leukemia progenitor cells, treatment with AT for 48 h induced a dose-dependent increase in apoptosis (Fig. 6B). These findings confirmed that, similar to cultured CML-BC cells, treatment with AT induces apoptosis of patient-derived, Bcr-Abl-expressing, CD34+ leukemia progenitor cells. Because of the limited sample size, it was not feasible to determine whether the mechanism of resistance against imatinib in these leukemia progenitor cells was gene amplification and overexpression of Bcr-Abl or point mutation in the ATP-binding region of Bcr-Abl (33, 40, 41). However, we investigated whether treatment with AT would also down-regulate the levels of Bcr-Abl carrying the previously described, imatinib resistance-associated point mutation, T315I (33, 40). This mutation contains the single nucleotide C-to-T change that results in a threonine to isoleucine substitution at position 315. We engineered a T315I mutation into the wt p210 bcr-abl and transfected the wt and the mutant construct into the human AML HL-60 cells, using the pSTAR ex-
pression vector, as above (27). Subclones of the cells were isolated that demonstrated a doxycycline-inducible expression of either wt or T315I mutation-containing Bcr-Abl (Fig. 6C). Exposure to 5 μg/ml of doxycycline plus 2 μM imatinib induced 80% apoptosis in HL-60/Bcr-Abl (wt) and <10% apoptosis in HL-60/Bcr-Abl (T315I) cells, as determined by Annexin V staining and flow cytometry (data not shown). Cotreatment with 5 μg/ml of doxycycline with 2.0 μM AT for 48 h clearly reduced the levels of the wt and T315I mutation-containing Bcr-Abl (Fig. 6C). Additionally, cotreatment with doxycycline and 2.0 μM AT for 96 h induced 35 and 38% apoptosis of HL-60 cells containing wt and T315I mutation-containing Bcr-Abl, respectively.

DISCUSSION

Historically recognized as the active ingredient in Fowler’s solution, which was used many decades ago for the treatment of CML, AT is currently approved for the treatment of all-trans retinoic acid-refractory APL as well as being tested against a variety of hematological and other malignancies (19, 42). AT is known to be a mito-
chondria-toxic agent that can induce the reactive oxygen species and mitochondrial permeability transition, release of cyt c into the cytosol, activation of caspase-3, and apoptosis (22). At the levels clinically achievable with doses higher than those used in APL (19, 42), AT has been demonstrated to reduce the intracellular levels of Bcr-Abl and exert a synergistic antileukemia effect with imatinib against Bcr-Abl-positive acute leukemia cells (23, 24). Attenuation of the levels of antiapoptotic Bcr-Abl by AT precedes and facilitates its cytotoxic effects (24). The present studies have elucidated the mechanism by which AT rapidly inhibits the translation and levels of Bcr-Abl, without attenuating its mRNA levels or promoting proteasomal degradation, thus inducing apoptosis of Bcr-Abl-expressing acute leukemia cells.

Treatment with AT rapidly inhibited the intracellular activity of S6K1. The in vitro inhibition of S6K1 by AT was modest and less than the in vivo inhibitory effect of AT on S6K1 activity. AT also inhibited the levels of p-S6K1. This suggested that inhibition of either the upstream mTOR or PDK1 kinase may be involved in the inhibition of S6K1 activity (32, 37, 38, 43). Our findings demonstrate that AT rapidly inhibits PDK1 and S6K1 activity without affecting its mRNA levels or promoting proteasomal degradation, thus inducing apoptosis of Bcr-Abl-expressing acute leukemia cells.

Treatment with AT rapidly inhibited the intracellular activity of S6K1. The in vitro inhibition of S6K1 by AT was modest and less than the in vivo inhibitory effect of AT on S6K1 activity. AT also inhibited the levels of p-S6K1. This suggested that inhibition of either the upstream mTOR or PDK1 kinase may be involved in the inhibition of S6K1 activity (32, 37, 38, 43). Our findings demonstrate that AT rapidly inhibits PDK1 and S6K1 activity without affecting mTOR. A recent report by Stolovich et al. (32) suggested that S6K1 activity is not involved in the translation of TOP-containing mRNA. In this report, the translational control of TOP mRNA was attributed to a PI3K- and PDK1-dependent pathway (32). It is noteworthy that AT mediated the decline in the levels of Bcr-Abl, as well as the associated decline in the levels and activity of phospho-AKT and the levels of phospho-4E-BP1, which occurred after the AT-mediated inhibition of PDK1 and S6K1 activities. This strongly suggests that the inhibitory effect of AT on PDK1 and S6K1 is upstream and could be mechanistically responsible for the effect of AT on Bcr-Abl, AKT, and 4E-BP1 (Fig. 7; Ref. 37). In Jurkat/Bcr-Abl (5’UTR-) cells, despite its inhibitory effect on the phospho-S6K1 and S6 protein levels, the inability of AT to reduce the intracellular levels of the 5’UTR-Bcr-Abl or AKT levels and activity clearly supports that AT-mediated inhibition of PDK1 and S6K1 is the lead molecular event that is responsible for the attenuation of Bcr-Abl, which inhibits AKT and the other downstream molecular events (Fig. 7). These observations raise the possibility that through this mechanism, i.e., by inhibiting PDK1 and S6K1, AT may also inhibit the translation of other, here-tofore unidentified, oncoproteins that may have a long and structured mRNA containing the TOP sequence.

Importantly, treatment with AT also down-regulated the levels of Bcr-Abl in fresh CD34+ leukemia blast progenitor cells from patients who had developed imatinib-resistant blast crisis of CML. This has been shown to be attributable to amplification and increased expression of Bcr-Abl or caused by specific point mutations in Bcr-Abl (33, 40, 41). In the present studies, we were unable to determine the precise mechanism responsible for imatinib resistance of the patient-derived CD34+ leukemia blast progenitor cells. However, our findings suggest that AT may at least be able to down-regulate the levels of a T315I mutation-containing Bcr-Abl (Fig. 6E). The effect of AT on a Bcr-Abl that is the product of the amplified bcr-abl gene remains to be determined. Significantly, treatment with 17-AAG, which attenuates intracellular Bcr-Abl by promoting its proteasomal degradation, has recently been shown to exert this effect in fresh leukemia blasts from patients who had developed imatinib-refractory blast crisis of CML (28). Collectively, these findings indicate that AT or 17-AAG may be useful in the treatment of imatinib-refractory advanced phases of CML. Clinical trials to investigate the activity of these agents have been developed or are on the drawing board (24).

Fig. 6. AT lowers Bcr-Abl and induces apoptosis of fresh leukemia cells. A, the effect of 2.0 μM AT for 48 h on Bcr-Abl levels in three fresh samples of CD34+ leukemia progenitor cells derived from patients with imatinib-refractory blast crisis of CML. Actin levels served as the loading control. B, AT (1.0, 2.0, or 5.0 μM for 48 h)-induced apoptosis of five fresh samples of CD34+ leukemia progenitor cells, as above. C, the effect of 2.0 μM AT and/or 5.0 μg/ml of Dox for 48 h on Bcr-Abl (wt) and Bcr-Abl (T315I) levels in HL-60/Bcr-Abl (wt) and HL-60/Bcr-Abl (T315I) cells, respectively. Actin levels served as the loading control.
The inhibitory effects of AT on PDK1 and S6K1 also has implications for the treatment of cancers and leukemia that have increased phosphorylation and activity of PDK1 and S6K1 through increased activity of the PI3K/AKT/mTOR signaling pathway (37, 43). Because this pathway is activated by multiple oncogenic mechanisms in a variety of tumors (37, 43), the targeted inhibition of increased PDK1 and S6K1 activity by AT, in addition to a number of other mechanisms responsible for its cytotoxic effects (44), may confer therapeutic benefit in these malignantities and needs to be investigated. S6K1 has been recently shown to signal not only cell growth but also promote cell survival by inactivating the pro-death molecule Bad (45). Therefore, treatment with AT may be especially relevant where a specific cytokine-induced pathway results in the activation of PDK1 and S6K1, as is the case for interleukin-6 in multiple myeloma (46). Collectively, the findings presented here underscore the need to identify additional leukemia and cancer types where targeting of PDK1 and S6K1 by agents such as AT would abrogate the main mitogenic and survival signaling and perhaps also down-regulate the pathogenic oncprotein. Additionally, because many of the key protein kinases involved in the mitogenic and survival signaling are also the client proteins chaperoned by heat shock protein 90, geldanamycin and its analogues may also have efficacy, in combination with AT, to promote down-regulation of the signaling molecules and inhibit the mitogenic and survival mechanisms in leukemia (47).

REFERENCES


Arsenic Trioxide Inhibits Translation of mRNA of \textit{bcr-abl}, Resulting in Attenuation of Bcr-Abl Levels and Apoptosis of Human Leukemia Cells
